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The Fate of the Aglucone Group in Aqueous-Chlorine Oxidation of Carbon-14 Labeled Methyl β-D-Glucopyranoside

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THE FATE OF THE AGLUCONE GROUP IN AQUEOUS-CHLORINE OXIDATION OF CARBON-14 LABELED METHYL β-D-GLUCOPYRANOSIDE

A thesis submitted by

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SUMMARY

Carbon-14 labeled methyl β-D-glucopyranoside was oxidized with aqueous chlorine at 25°C. and pH 7 to determine the origin of some of the smaller oxidation products. Particular attention was paid to those products which came from the aglucone group, but both aglucone-labeled glucoside and glucosyl-randomly-labeled glucoside were oxidized. Isotope dilution techniques were used in the analyses for unreacted glucoside, glucose, arabinose, methanol, formaldehyde, formic acid, and for methanol which could be formed by hydrolysis from other products. The specific activity of the isolated and purified, isotopically diluted products (or derivatives of the products) was determined by wet combustion to carbon dioxide by Van Slyke's technique, followed by measurement of the specific activity of that carbon dioxide in Bernstein-Ballentine proportional counting tubes. Yields of the products were calculated from this specific activity data. Carbon dioxide, as an original oxidation product, was determined without dilution by making use of its radioactivity.

The main oxidative attack was shown to be on the glucosyl unit, because only 5.7% of the aglucone carbon from the glucoside which had been oxidized was accounted for as aglucone oxidation products. These oxidation products were: formaldehyde, which contained 4.7%, formic acid, which contained 0.6%, and carbon dioxide, which contained 0.4% of this aglucone carbon. The attack on the glucosyl portion of the glucoside was accompanied by the release of methanol into the solution, which was found to account for 38.4% of the aglucone carbon from the oxidized glucoside. Most of the remaining aglucone carbon was found by hydrolyzing the reaction products and redetermining the methanol in the solution. This showed that most of the remaining aglucone carbon must have remained on an oxidized glucosyl residue, and thus much of the oxidative attack is not on the Carbon 1-aglucone portion of the glucoside, but is on the remainder of the glucosyl group.

In these oxidations, about one per cent of the starting material was found as arabinose and one-half per cent as glucose when 22.4% of the starting material had been oxidized. Finding this glucose showed that the aglucone methyl group of the methyl β-D-glucopyranoside was the site of some oxidative attack to give formaldehyde and glucose. However, the manner in which the yield of formaldehyde increased during the oxidation showed that this attack on the aglucone methyl group was not the only method by which formaldehyde containing aglucone carbon was produced. Some of the free methanol in solution may have been oxidized to formaldehyde, or a methoxyl (or an oxidized methoxyl) group, still bonded to some of the primary reaction products, may have been converted to formaldehyde. The relative amounts of methanol, formaldehyde, formic acid, and carbon dioxide produced during the oxidation does show that methanol was not rapidly oxidized to carbon dioxide via formaldehyde and formic acid.

Over twenty times as much glucosyl carbon as aglucone carbon was oxidized to carbon dioxide in this study. Of the starting glucosyl carbon, 1.99% was found as carbon dioxide, while only 0.089% of the aglucone carbon was found as carbon dioxide. Only a trace of glucosyl carbon (0.14%) was found as methanol.

The oxidant consumptions (milliequivalents of oxidant consumed per millimole of glucoside oxidized) for two similar oxidations of aglucone-labeled glucoside were the same, but oxidant consumptions for identical oxidations of aglucone-labeled and glucosyl-labeled glucosides did not agree because the results from the analyses for unreacted glucoside did not agree. There is a possibility that an isotope effect during the oxidations caused this difference, but the effect was not used for mechanism studies because the specificity of the oxidative attack was not known and because experimental error masked the effect.

When the oxidant consumption by the glucoside and its products was followed during the oxidations, corrections had to be made for the loss of oxidant through disproportionation into chlorate and chloride. This study pointed out the importance of making this correction, especially in neutral or nearly neutral solutions.

The results of this study were consistent with accumulating evidence in the literature, that at pH 7 the oxidative attack is somewhat specific for carbon atoms two and three.

INTRODUCTION

BACKGROUND

Hypochlorite bleaching of wood pulp, if improperly controlled, can result in an undesired attack on the cellulose in addition to the desired attack on the lignin (1). This attack reaches a maximum at pH 7 (2-5). In studying this attack, both cellulose and model compounds for cellulose have been used. Methyl β-D-glucopyranoside (abbreviated MBG) has been the model compound used most often (6-9). A discussion of the work in this field will include some studies that have only followed the rate of oxidant consumption or the production of certain functional groups, and will include others in which oxidation products have been identified and determined. There have been several careful studies of the oxidation products formed, although the conditions for these oxidations varied widely.

In an early work on the oxidation of MBG (6), Dyfverman, Lindberg, and Wood mentioned that reactions occurring in the aglucone were disregarded in such studies. Their statement was still true at the beginning of this present study. These authors passed gaseous chlorine through an aqueous solution of MBG and kept the solution saturated. They followed the reaction with optical rotation measurements and chromatography, then isolated gluconic acid from the reaction solution and concluded that 50% of the MBG formed gluconic acid in 14 days. Gluconic acid and 5-ketogluconic acid, both as the free acids and as the lactones, were found chromatographically. Chromatograms from similar experiments continued for 40 days, showed that the solution contained both gluconic acid and 5-keto-gluconic acid, but the proportion of keto acid was higher than in the solution from the 14-day oxidation. This indicated that the initial product was gluconic acid, which was in turn oxidized to the keto acid.

The maximum hydrochloric acid concentration during their experiments was about two normal. Tests with MBG in 2N hydrochloric acid showed no hydrolysis. The workers therefore concluded that the oxidation of MBG in aqueous chlorine, even in systems as acidic as theirs, did not proceed through an initial hydrolysis followed by attack upon the glucose residue.

In an extension of this work, the oxidation of methyl β-cellobioside in an aqueous chlorine system at room temperature and at about pH 1 was studied (10). Generally, methyl β-cellobioside reacted similarly to MBG. Cellobionic acid was formed, which in turn gave gluconic acid. Possibly, ketocellobionic acids were also present. As in the above work on MBG, methyl β-cellobioside and cellobionic acid were treated with 2N hydrochloric acid. This test showed that there was no initial hydrolysis of glucosidic bonds.

Several workers have studied various aspects of carbon dioxide production. From cellulose which had been oxidized in an acidic or a neutral medium, Kaverzneva (11) found that small amounts of carbon dioxide were given off during very mild saponification. She suggested that this carbon dioxide came from a carbonate ester group at Carbon 1, which had been formed during the oxidation (Fig. 1). She also worked with the various functional groups formed when cellulose is oxidized at different pH values.

Carbon dioxide was also found by Henderson when he oxidized MBG with aqueous chlorine at pH 4.5 (8). He thought that it may have come, in part, from the aglucone carbon or from the Carbon 1 position of the glucosyl group. Arabinose and glucose were formed in a ratio of about 2:1. Oxalic acid, 2-ketogluconic acid, and 2,5-diketogluconic acid were also products. The glucose formed was not a product of the direct hydrolysis of MBG but was the product of an "oxidative

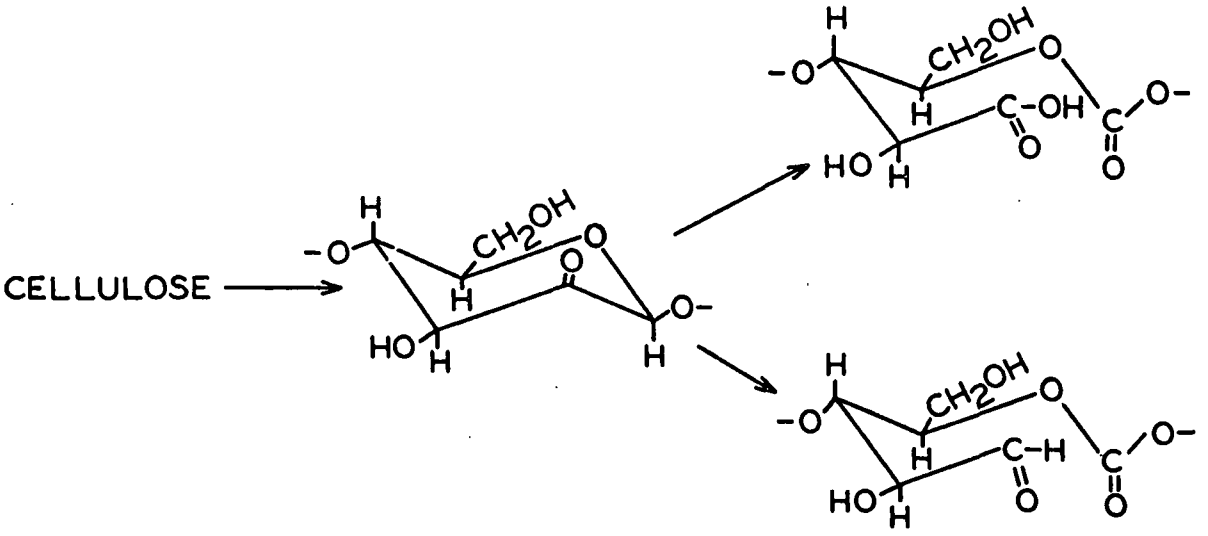

Figure 1. Formation of Carbonate Ester Groups in Oxidized Cellulose, from (11)

hydrolysis." By oxidative hydrolysis, he referred to either a reaction which involves the simultaneous oxidation and hydrolysis of the glycosidic bond or to a pair of reactions involving the oxidation of the glucosyl or the aglucone group, followed by the hydrolysis of the glycosidic bond. After finding that arabinose did not come from 2-ketogluconic acid, and probably not from glucose or gluconic acid, Henderson concluded that the arabinose was produced by direct oxidation of the MBG. The Carbon 1 carbonate ester could have been an intermediate in this pathway.

There was too little oxalic acid formed to trace its source. Under his oxidation conditions, cotton behaved in a manner similar to the MBG.

Daniel (12) extended the study of the carbonate ester group using cellulose-(^{14}C) specifically labeled in the Carbon 1 and Carbon 6 positions through the action of Acetobacter xylinum. He oxidized the cellulose with aqueous chlorine at pH 4.5 and room temperature, and concluded that not more than 21% of the carbon dioxide obtained upon saponification came from the Carbon 1 position.

He qualitatively confirmed Henderson's finding of D-arabinose, but found no more than 0.2%, whereas Henderson found 1%. Daniel suggested that arabinose may be formed from end group oxidation and/or oxidative cleavage of the Carbon 1-Carbon 2 bond of the anhydroglucose, leaving Carbon 2 as an aldehyde group. Erythrose and xylose were also products of the oxidation.

One of the most complete studies of the oxidation of MBG and related compounds has been done by Theander (7, 13) who oxidized MBG with aqueous chlorine solutions at various pH values. He searched mainly for neutral oxidation products and found, depending on the pH during the oxidation, from 1.6 to 4.0% of the MBG had been oxidized to oxoglucosides (2-keto-MBG, 3-keto-MBG, 4-keto-MBG, and 6-aldehydo-MBG). The maximum yield of these neutral compounds was obtained at pH 4, but the maximum rate of oxidation was at pH 7, as it is for the attack on cellulose by aqueous chlorine. Ribulose, glucosone, gluconic acid, glyoxylic acid, erythronic acid, glucose, and arabinose were also found. He suggested that ketoglucosides may be intermediates in the formation of glyoxylic and erythronic acids. The ratio of glucose to arabinose varied from 1.6/1 to 3.1/1. This is in conflict with Henderson's results which showed twice as much arabinose as glucose.

Methyl 4-O-methyl-β-D-glucopyranoside is an even better model compound for cellulose than is MBG because both the Carbon 1 and the Carbon 4 positions are blocked. Also, the components of starch (amylose and amylopectin) are somewhat similar to cellulose because they contain anhydroglucose units, although it must be remembered that the linkages between units are α instead of β.

In their studies of carbohydrate oxidation, Whistler and co-workers (14-16) have used these and similar compounds. Products suggesting a Carbon 2-Carbon 3

cleavage were found when both amylose and methyl 4-O-methyl-β-D-glucopyranoside were oxidized at pH 9 and 11 at 25°C. Evidence that at pH 9 the oxidative action of hypochlorite on amylose is not random, but is extensively specific for the Carbon 2 and the Carbon 3 positions, was found. D-Erythronic acid and its lactone, and glyoxylic acid were among the products found. These workers state that depolymerization of the amylose might be expected if a carbonyl group is formed at the Carbon 2 or the Carbon 3 position of the sugar units, for its presence would weaken the link at the Carbon 1 position.

From the oxidation of methyl 4-O-methyl-β-D-glucopyranoside at pH 9 and 25°C., they isolated what they thought to be the disodium salt of 2-O-methyl-3-O-(glyoxylic acid methyl acetal)-D-erythronic acid (Fig. 2). They also isolated glyoxylic acid and glyoxal. These products were evidence for a scission between Carbon 2 and Carbon 3. Glyoxal could also be evidence for a Carbon 3-Carbon 4 cleavage (see below).

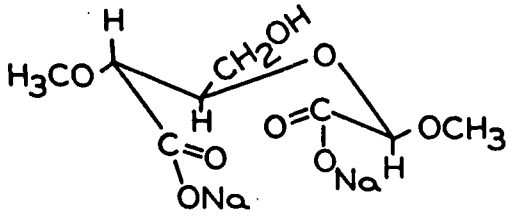

Figure 2. Disodium Salt of 2-O-Methyl-3-O-(glyoxylic acid methyl acetal)-D-erythronic Acid

The production of more glyoxal from methyl 2-O-methyl-α-D-glucopyranoside than from the 4-O-methyl glucoside supported their belief that the glyoxal came from a 3-carbon fragment, 2-carboxy glycolic aldehyde, which in turn was derived from the oxidative cleavage of a 3,4-enediol structure in the sugar ring.

In the oxidation of amylopectin with hypochlorite at 25°C. and pH 3-12, principal products, regardless of pH, were glyoxylic acid and erythronic acid,

with a maximum yield at pH 7. This suggests that hypochlorite cleaves the Carbon 2-Carbon 3 bond at all pH values, but is most specific near pH 7. In this work they found that the chlorate formed in the solution is not an oxidant for amylopectin, nor for hypochlorite-oxidized amylopectin, at the pH values examined of 3, 5, and 7.

Various kinetic studies on the oxidation of carbohydrates by aqueous chlorine have been made. The widely varying results (although it must be admitted that conditions were different in each case) show that there is still much to be learned about this oxidation.

The formation of functional groups, oxygen consumption, change in D.P., and rate of oxidation in the hypochlorite oxidation of cotton at pH 5-10 at 25°C. was studied by Epstein and Lewin (4). They found a maximum rate at pH 7. By comparing the rates of oxidation with the relative amounts of hypochlorous acid and hypochlorite ion present at each pH, they concluded that the rate was proportional to $(HOCl)^2(OCl^-)^{1/2}$. Correcting for the "concentration of cotton" and chloride ion, they derived the rate equation

$$\frac{dc}{dt} = -k(HOCl)^2(OCl^-)^{1/2}(cotton)^{1/2}/(Cl^-)^{1/2} \qquad (1).$$

Different kinetics were found by Wolfrom and Lucke (17) for the oxidation of methyl β-cellodŏligosides (D.P. 1-5) and cellodŏligoses. Working with sodium hypochlorite at pH 9 at 50.5°C., they found two successive second-order reactions which were zero order in substrate and second order in hypochlorite. They did not find the second reaction at 43°C. The oxidation products suggested a cleavage of glycol units. Their results were consistent with Herbst's proposal (18) that an intermediate compound in the decomposition of hypochlorite was also the oxidizing agent.

A third set of kinetic results was obtained by Grillo (9) in his oxidation of glucose, glucono-δ-lactone, MBG, and four other glucosides with aqueous chlorine at 35.7°C. He found the order in MBG to be 1.10, 0.84, and 0.56 at pH 2.1, 4.6, and 6.0, respectively, and considered the 0.56 value to be a significant departure from unity. At pH 6, the reaction was first order in oxidant, with a large dependence on the initial oxidant concentration. He thought this dependence on initial concentration indicated that OCl^- is a catalyst. Using buffers, he varied the pH from 2.1 to 6.5. He followed the rate of oxidant consumption during the oxidations, varying conditions so that rate constants for oxidation by molecular chlorine and by hypochlorous acid, and the catalytic constants for certain ionic species from both the oxidant and the buffers, could be calculated. In the Results and Discussion section, his rate will be compared with the rate found in this study.

The work discussed in the above articles has done much to explain the reactions which cellulose, model compounds for cellulose, and polysaccharides undergo during oxidation in aqueous chlorine. The mechanism is not yet fully understood, nor have all the oxidation products been carefully studied, particularly the products from the aglucone group of the simple glucoside model compounds.

There is a lack of agreement about the mechanism of the oxidation. Lichtin and Saxe (19) suggested a complex between the glycosidic oxygen and the halogen, which provides a role for base catalysis. Wolfrom and Lucke (17) suggest that an intermediate such as $Cl_2O_2^{--}$ or $Cl_2O_2^-$ may be the oxidant in their slightly basic system (pH 9). Epstein and Lewin (4) suggested a free radical mechanism. Also, results of the work on bromine oxidations may be applicable in understanding similar chlorine oxidations. A recent study of this type by Swain, Wiles, and Bader (20) involved the use of the deuterium isotope effect to establish

that the mechanism of the oxidation of alcohols by bromine water (pH 1-3) is one of hydride transfer from the carbon, followed by fast proton removal from the oxygen. This is related to a study by Friedberg and Kaplan (21) who oxidized tritium-labeled compounds, α and β glucose-1-t, with bromine in a barium carbonate buffer at 0°C. From the isotope effect they found, they concluded that the rate-determining step was the rupture of the carbon-hydrogen bond. The reactivity in these studies on carbohydrates may be related to the fact that there are three carbon-hydrogen bonds on one side of the glucose ring which are relatively unhindered sterically. Wells (22) has studied the reactivity of various carbohydrates and related their reactivity to this type of steric hindrance in the molecule.

Some mention will be made of these mechanisms in the Results and Discussion section, even though the present work was not a direct study of such mechanisms.

The purpose of the present study was to obtain additional information about these oxidations through the use of a powerful analytical tool — isotope dilution. Particular attention was paid to some of the smaller oxidation products, especially the ones originating in the aglucone group.

ISOTOPE DILUTION

Frequently, quantitative analysis for a component of a mixture is needed where no quantitative method of isolation is known. While it may be possible to separate a particular compound from a complex mixture with satisfactory purity, the yield from the separation method may be low and unknown. In such a case, the analysis may often be made using isotope dilution techniques (23). The solutions obtained from aqueous chlorine oxidation of carbohydrates fall into such a category. For example, the solids in the oxidation solutions obtained in this study

were about 95% inorganic salts and 4% unreacted MBG, with the many oxidation products accounting for the remaining 1%.

The validity of isotope dilution analyses depends upon the fact that, qualitatively, isotopes of the same element are indistinguishable in chemical behavior. Ordinary processes of distillation, crystallization, and chromatography effect no significant fractionation of isotopes (24). However, differences in isotopic composition can cause differences in the reaction rates. The effect of this difference in rate will be discussed later.

Because the isotope of carbon with an atomic mass of 14 is the isotope used in this study and is now rather commonly used in organic chemistry, the discussion of isotope dilution techniques will be limited to carbon-14, (^{14}C). Carbon-14 is radioactive, slowly disintegrating into nitrogen and beta rays.

$$^{14}_{6}C \longrightarrow \beta + {}^{14}_{7}N \qquad (2).$$

The half life is 5700 years, and the beta particles (electrons) have an energy of 0.155 mev. (25). With this long half life there is no correction to be made for the decrease in activity during the course of the experiment. The low energy of the electrons emitted allows experiments to be made without elaborate safety measures. Usually, ordinary laboratory techniques may be employed. The beta rays provide a convenient means of measuring the amount of this abnormal isotope present.

It is not implied, when one speaks of isotopically labeled compounds, that all of the carbon atoms in the compound are carbon-14. The two batches of MBG oxidized in this study had about 1.6 and 9 atoms of carbon-14 per million carbon atoms. Even at this dilution, there were approximately 50,000 disintegrations per minute per milligram of glucoside for the latter compound.

Isotope dilution may be carried out by two methods. To an unknown mixture of unlabeled compounds may be added a known weight of a compound to be determined containing a known amount of isotopic carbon. This compound is separated, purified, and its activity redetermined. By comparing the new and the original activity, the amount of the inactive compound in the mixture may be calculated. The second method is similar, but starts with a mixture of labeled compounds and dilutes them with unlabeled compounds. The latter method was used in this study.

The first step (dilution) and the last step (redetermination of the activity of the isolated compound) must be quantitative in isotope dilution analyses. All intermediate steps need only have a yield sufficiently high to give workable quantities. For example: If an MBG oxidation solution contains an unknown amount, X, of glucose of specific activity A (known from the activity of the starting MBG), a known amount, Y, of unlabeled glucose is added. This first step is quantitative. The glucose may then be separated by any convenient means such as extraction, ion exchange, chromatography, crystallization, etc., without these steps being quantitative. After pure glucose has been separated (a few milligrams is sufficient), the specific activity is redetermined and found to be B. It may be shown that

$$X = \frac{YB}{A - B} \qquad (3).$$

The unknown amount of glucose in the solution has therefore been determined. This is the basic calculation in all the isotope dilution analyses in this study.

Of course, use may be made of the radioactivity of an oxidation product without the dilution step, providing a quantitative method of separating that particular product is available. The total amount of activity found is then a

measure of the amount of the product. This method was used for carbon dioxide analysis in this study.

ISOTOPE EFFECT

The interpretation of results when using isotopes depends upon the assumption that isotopes of the same element are qualitatively indistinguishable. This is not true quantitatively, because in reactions there may be an isotope effect. Isotopic atoms in a molecule may cause a change in the reaction rate or in the position of equilibrium. Generally, but not always, the lighter molecule reacts faster than the heavier one (24). For the series: hydrogen, deuterium, tritium, which have weight ratios of 1:2:3, this effect can be quite large, even a factor of ten or more. For the carbon-14 used in this study, with a weight ratio between it and ordinary carbon of only 12:14, the effect is not nearly so large. It was concluded that the isotope effects in this study would not have affected the results by more than $\pm$ 10%. Appendix I gives the reasons for reaching this conclusion.

EXPERIMENTAL TECHNIQUES AND RESULTS

MEASUREMENT OF SPECIFIC ACTIVITY

All determinations of specific activity in this study involved converting the samples to carbon dioxide, followed by measuring the radioactivity of that carbon dioxide. Weighed solid samples were converted to carbon dioxide in the Thomas-Van Slyke manometric apparatus. Samples already in the form of carbon dioxide could also be transferred into this apparatus. After measuring the pressure, volume, and temperature of the carbon dioxide, the amount of carbon in the sample was calculated; usually this amount was already known from the weight of the sample. The carbon dioxide was then transferred into a Bernstein-Ballentine proportional counting tube. The tube was then attached to a Nuclear-Chicago model 182 scaling unit where the radioactivity of the carbon dioxide was determined.

By dividing the activity, disintegrations per minute, found in the Bernstein-Ballentine tube by the milligrams of carbon, found either by weight of sample or in the manometric apparatus, the specific activity of the sample could be found. Specific activity in this study is expressed as disintegrations per minute per milligram of carbon, dis./(min.)(mg. C).

The over-all accuracy of this procedure in this study was about $\pm$ 2%. Appendix II gives more detailed information on the above procedures.

PREPARATION OF THE STARTING MATERIAL

A high-yield, three-step synthesis of methyl β-D-glucopyranoside from glucose was used. Glucose(^{14}C) or methanol(^{14}C) was used in some of these syntheses, and in both cases high yield was desirable.

Glucose was converted to 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (ABG). This compound was converted to methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (MTABG), which was deacetylated to give methyl β-D-glucopyranoside (MBG) Fig. 3.

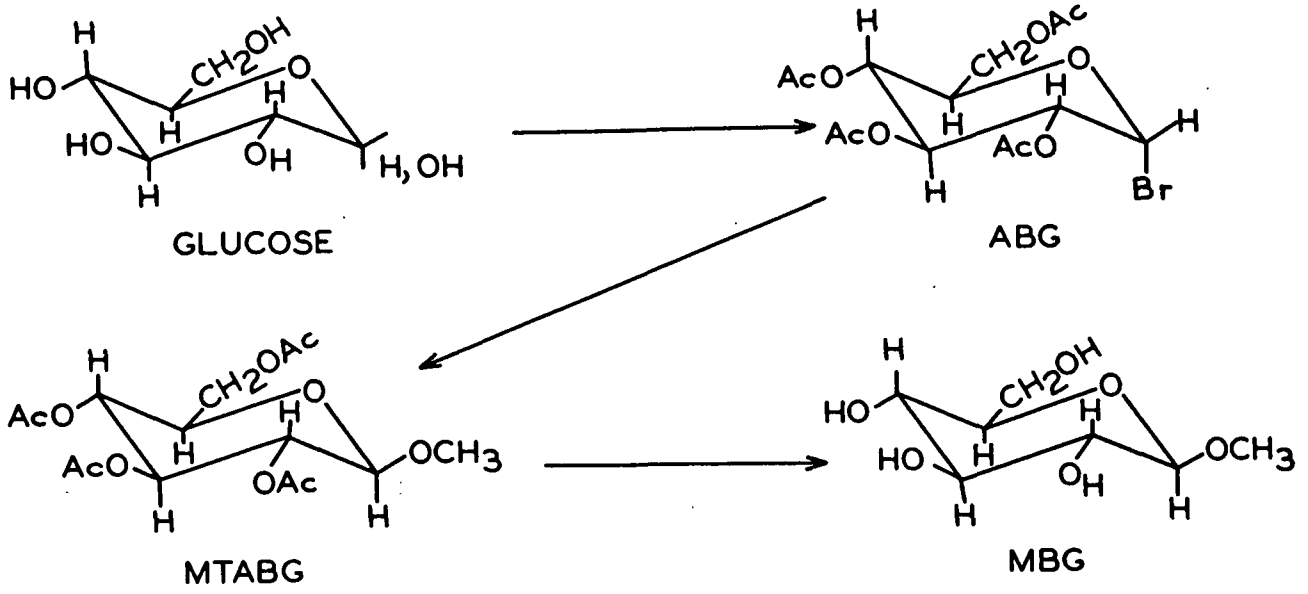

Figure 3. Synthesis of Methyl β-Glucoside

PREPARATION OF ACETOBROMOGLUCOSE

The procedure used in preparing 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (acetobromoglucose, ABG) from glucose is essentially that of Bárczai-Martos and Körözy (26). This procedure converts glucose to glucose pentaacetate in an acetic anhydride solution with perchloric acid as catalyst. Hydrogen bromide is then formed in the solution through the addition of red phosphorus, bromine, and water. This hydrogen bromide converts the glucose pentaacetate into ABG.

The only departure from the published method was in isolating the product. At the end of the synthesis, both the chloroform layer containing the ABG and

the acidic aqueous layer were filtered prior to separation. This filtering removed the remaining red phosphorus and made the subsequent separations easier. Also, to obtain the highest possible yields, the separated aqueous layer was back-extracted with chloroform an additional time.

The crude product could be used as a chloroform solution or pure product could be isolated by crystallization. After recrystallization, the product could be kept for several weeks in a vacuum desiccator without decomposition.

Representative yields for this synthesis were 89.0, 76.8, and 81.8%. Representative melting points of the first crops of crystals were 87.5-88.5°C. and 85.0-88.0°C. compared with the literature value of 88-89°C. (27). Optical rotation, $[\alpha]_D$, was +191.1° (c 2, chloroform) compared with the literature value of +197.8° (27).

PREPARATION OF METHYL β-D-GLUCOSIDE TETRAACETATE

The method of Reynolds and Evans (28) was used for the synthesis of MTABG from the ABG. Their work was for the preparation of gentiobiose octaacetate. In this study their method was modified by substituting methanol for their β-D-glucose tetraacetate. The product is then MTABG.

The reaction is a Koenigs-Knorr reaction carried out in an opaque reaction vessel in a chloroform solution. Nonindicating Drierite is used to keep the solution anhydrous. Silver oxide is added as an acid accepter, and iodine is added as a catalyst. A fourfold excess of methanol was used in most of the syntheses. However, using the stoichiometric amount in the case of methanol(^{14}C) caused no decrease in yield. Crystals were obtained upon cooling, after dissolving the concentrated sirup from the synthesis in hot absolute ethanol. The product was recrystallized from ethanol.

Representative yields were 91.4, 90.7, and 84.7% based on ABG. Melting points varied from 100.5-104.5°C. to 104.5-107.5°C. for various runs compared with a literature value of 104-105°C. (29). Optical rotation, $[\alpha]_D$, varied from -21.6 to -24.1° (c 2, ethanol) compared with literature values of -22.2° (29) and -27.2° and -24.6° (30).

PREPARATION OF METHYL β-D-GLUCOSIDE

Deacetylation of MTABG was done in methanol using 1/400 of the theoretical amount of sodium methylate as the catalyst. The reaction was carried out in a refrigerator, allowing 36 hours for the deacetylation. After crystallization of the MBG, chromatographic analysis using Whatman No. 1 chromatographic paper and developer A (Appendix III) showed the first crop of crystals contained only MBG, glucose, and occasionally traces of two additional contaminants. From observations of the density of the chromatographic spots, less than 0.5% of the product was glucose.

MBG crystallizes[1] rather well from a mixture of alcohol and water by evaporation of the solvents. Crude MBG dissolved in an ethanol-water mixture recrystallized to give an 83.3% yield, with the product showing traces of glucose and two other contaminants by chromatographic examination. The size of the chromatographic spots indicated that the purity must have been about 99.9%. The melting point was 108-109°C. and the optical rotation, $[\alpha]_D$, was -34° (c 2, water) (weight corrected for hemihydrate water). Literature values vary considerably; both the above melting point and rotation agree with the higher values.

[1] MBG crystallizes as the hemihydrate $(MBG)_2 \cdot H_2O$. A Karl Fischer analysis for water showed 4.595 and 4.584% water (theoretical 4.43%).

An infrared spectrum of a sample of this MBG was identical with a spectrum of MBG synthesized by Church (31) by refluxing glucose with methanolic HCl and separation of the anomers.

PURIFICATION OF METHYL β-D-GLUCOSIDE

The labeled MBG used in a preliminary oxidation contained traces of glucose. The chromatographic purity was estimated to be 99.0 to 99.5%. Because glucose is one of the reaction products in this study, it is desirable that the starting material contain none of it.

The different crystalline crops of impure glucoside[1] were combined (5.582 grams total) and dissolved in six milliliters of 1N sodium hydroxide. The solution was heated to 80°C. for twenty minutes. As the impurity (glucose) was converted to saccharinic acids, the solution changed from colorless to amber. The solution was cooled and passed slowly through a column containing 12 milliliters of MB-3 monobed ion exchange resin.[2] Both sodium ions and the saccharinic acids were removed. The solution was then concentrated to a thick sirup.

Thirty milliliters of hot n-propanol and two milliliters of water were added. Fine crystals were obtained upon cooling (4.802 g.). The recovery was 86.1%. The crystals were chromatographically pure in three different developers (Appendix III). As little as 0.02% glucose could have been detected. The specific activity was statistically the same as before purification.

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[1] The example used for this discussion was the MBG* used in Oxidation 6.

[2] Amberlite MB-3 monobed resin is a mixture of Amberlite IR-120 and IRA-410 resins. These are all products of Rohm and Haas Co., Philadelphia, Pa., and obtainable through Mallinckrodt Chemical Works, St. Louis, Mo.

The optical rotation of a water solution containing 2.00 grams of anhydrous, purified glucoside per 100 milliliters was measured at different wavelengths. Table I shows the results.

TABLE I

OPTICAL ROTATION OF METHYL β-D-GLUCOSIDE

Light Source	Wavelength, mμ	$[\alpha]_D$,° [a]
Sodium lamp	589	-34.0
Mercury lamp (green line)	546	-40.5
Mercury lamp (blue line)	436	-67.3

[a] (c 2, water), at room temperature. Calculated as anhydrous MBG. Literature values for $[\alpha]_D$ (589 mμ) vary from -32 to -34°.

OXIDANT PREPARATION AND EQUILIBRATION

During the preliminary work, it was seen that loss of "available chlorine"[1] (Cl_2, HOCl, OCl^-) in the aqueous chlorine solutions was due to two factors: oxidation of carbohydrates, and disproportionation of the oxidant into chloride and chlorate. In making up a fresh chlorine solution, diluting, adjusting the pH, and immediately adding the MBG would give a system in which a little of the available chlorine was being consumed by the MBG and a much larger share was undergoing disproportionation. It would be nearly impossible, under these circumstances, to get duplicate runs. It was desirable that all the runs be carried out under the same conditions, otherwise the results for the various oxidations would not be directly comparable.

[1] Griffin (32) defines "available chlorine" as material which has the same oxidizing power as free chlorine, $Cl_{2(g)}$.

To avoid this problem as much as possible, a fresh chlorine solution was made up for each oxidation, diluted, and the pH adjusted. It was then allowed to approach equilibrium in the opaque reaction flask at 25°C. with the pH being kept at 7 automatically. Samples were periodically withdrawn by syringe and titrated with thiosulfate to determine the available chlorine content. After the solution had attained a comparatively stable available chlorine concentration, the MBG was injected. Figure 4 shows the manner in which the available chlorine concentration changed during these equilibrations. Appendix IV discusses aqueous chlorine systems in more detail and gives the methods used in this study for analyzing these systems.

After the oxidations, all the remaining available chlorine in the solutions was destroyed, but much chloride and chlorate remained. At no time did available chlorine reappear in these neutral oxidation solutions. This was evidence that chloride and chlorate were not recombining in a reverse of the disproportionation process.[1] Because there is less driving force for a reversal of the disproportionation process during the oxidation when some available chlorine was present than after the oxidation when none was present, it was concluded that there was no such reversal during the oxidation itself.

Before the MBG was injected into the oxidant, a sample of the oxidant was transferred by syringe into a smaller opaque flask in the same 25°C. bath. Then, during the oxidation, by periodically taking samples from this blank solution and the oxidation solution, the consumption of oxidant by the MBG could be calculated. This procedure was followed for the last three oxidations, two of which were excellent duplicates. Figures 5 to 7 show the results.

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[1] Available chlorine did reappear under strongly acidic conditions and at higher temperatures, as discussed in Appendix IV.

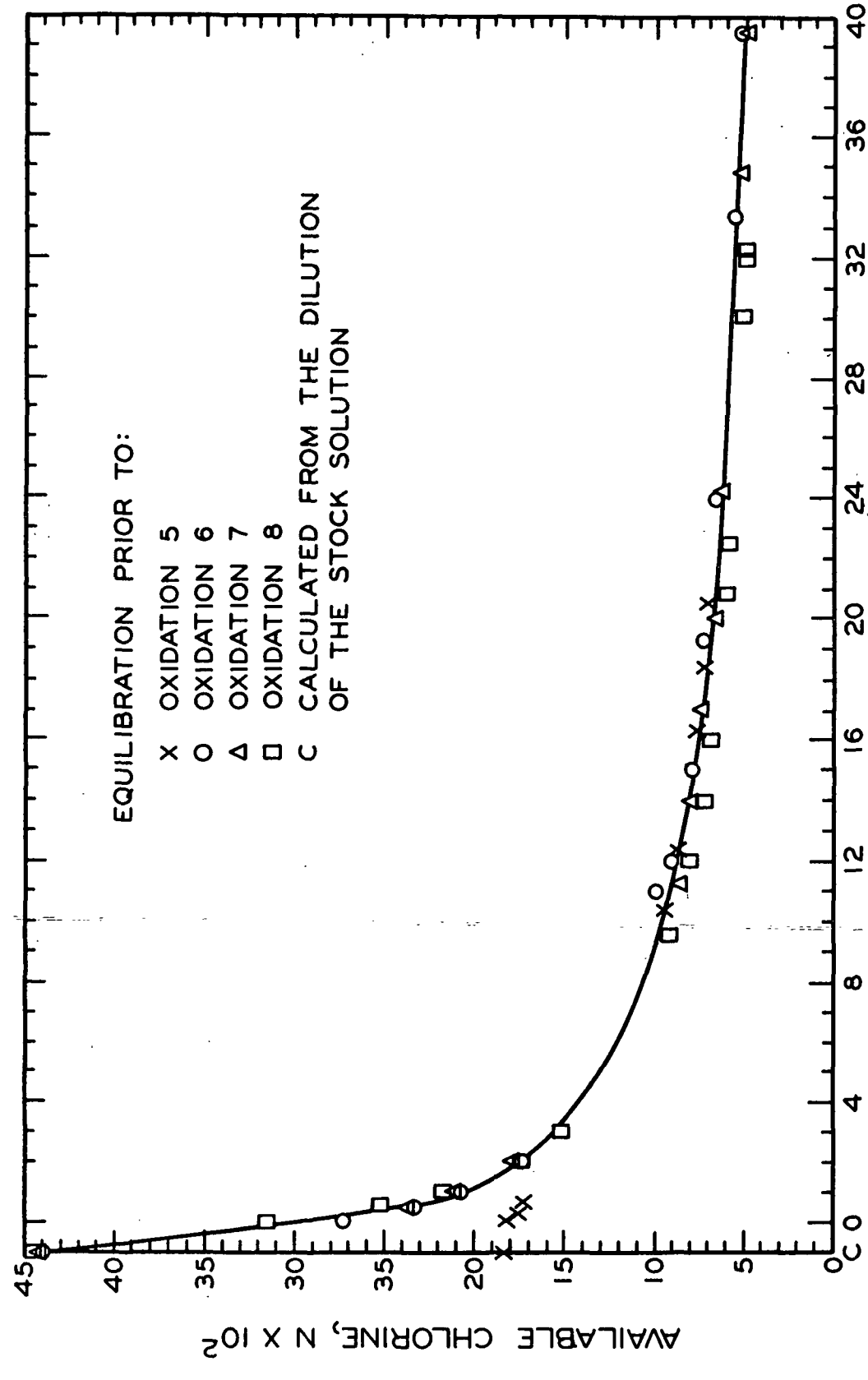

Figure 4. Equilibration of Aqueous Chlorine Solutions, pH 7, 25°C.

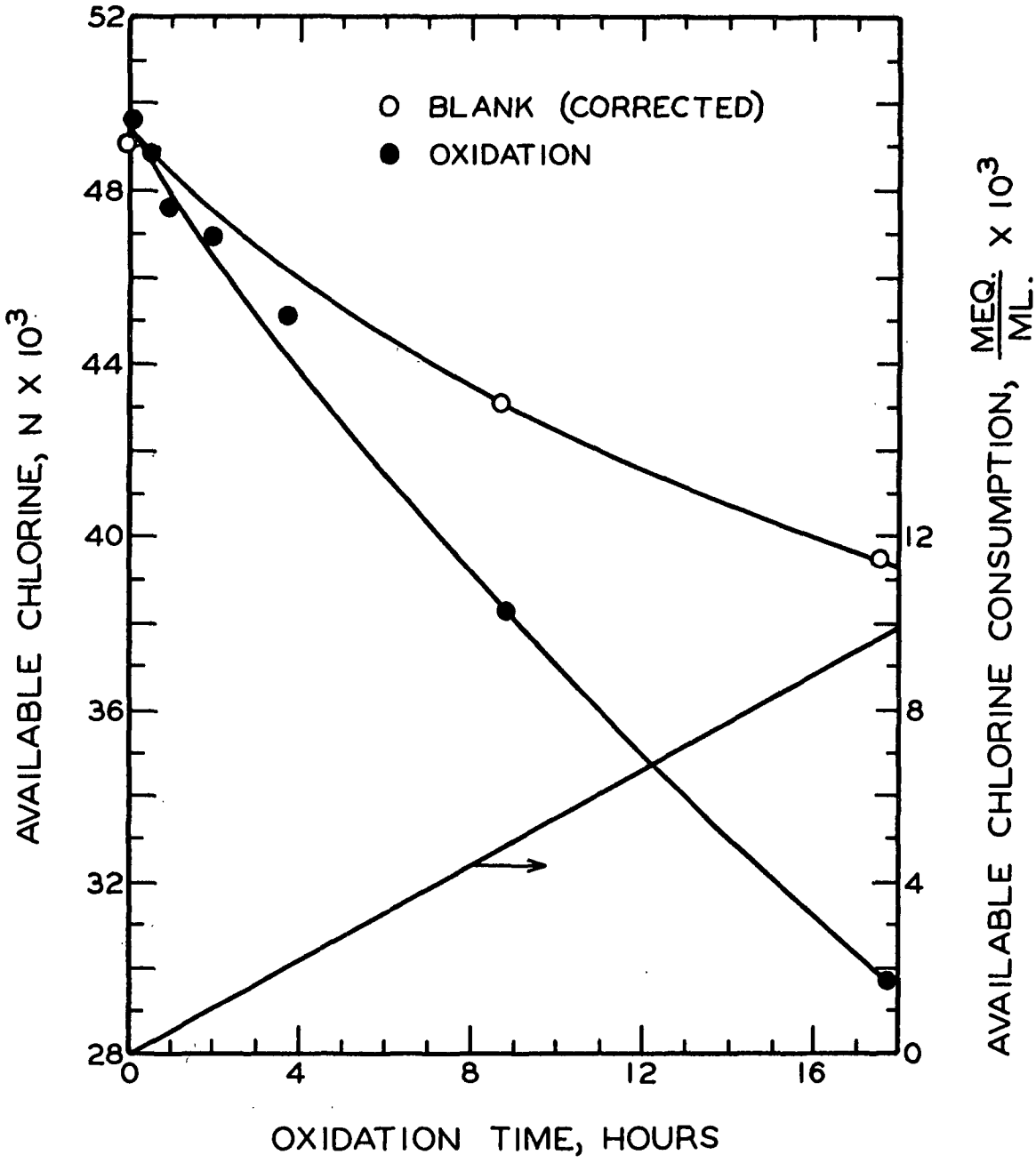

Figure 5. Concentration and Consumption of Available Chlorine During the Oxidation of Methyl β-D-glucopyranoside(glucosyl-^{14}C) (Oxidation 6)

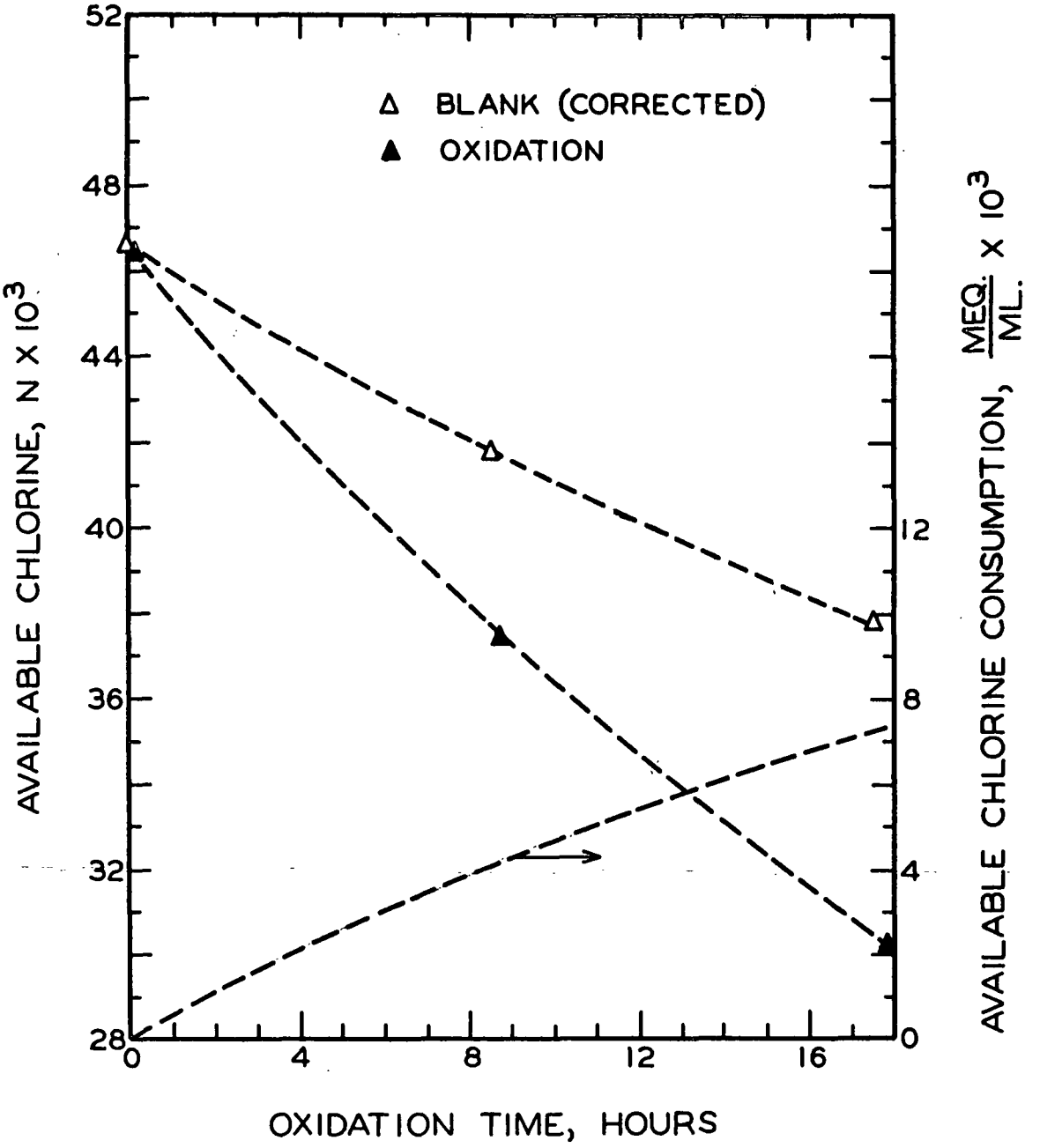

Figure 6. Concentration and Consumption of Available Chlorine During the Oxidation of Methyl β-D-glucopyranoside(aglucone-^{14}C) (Oxidation 7)

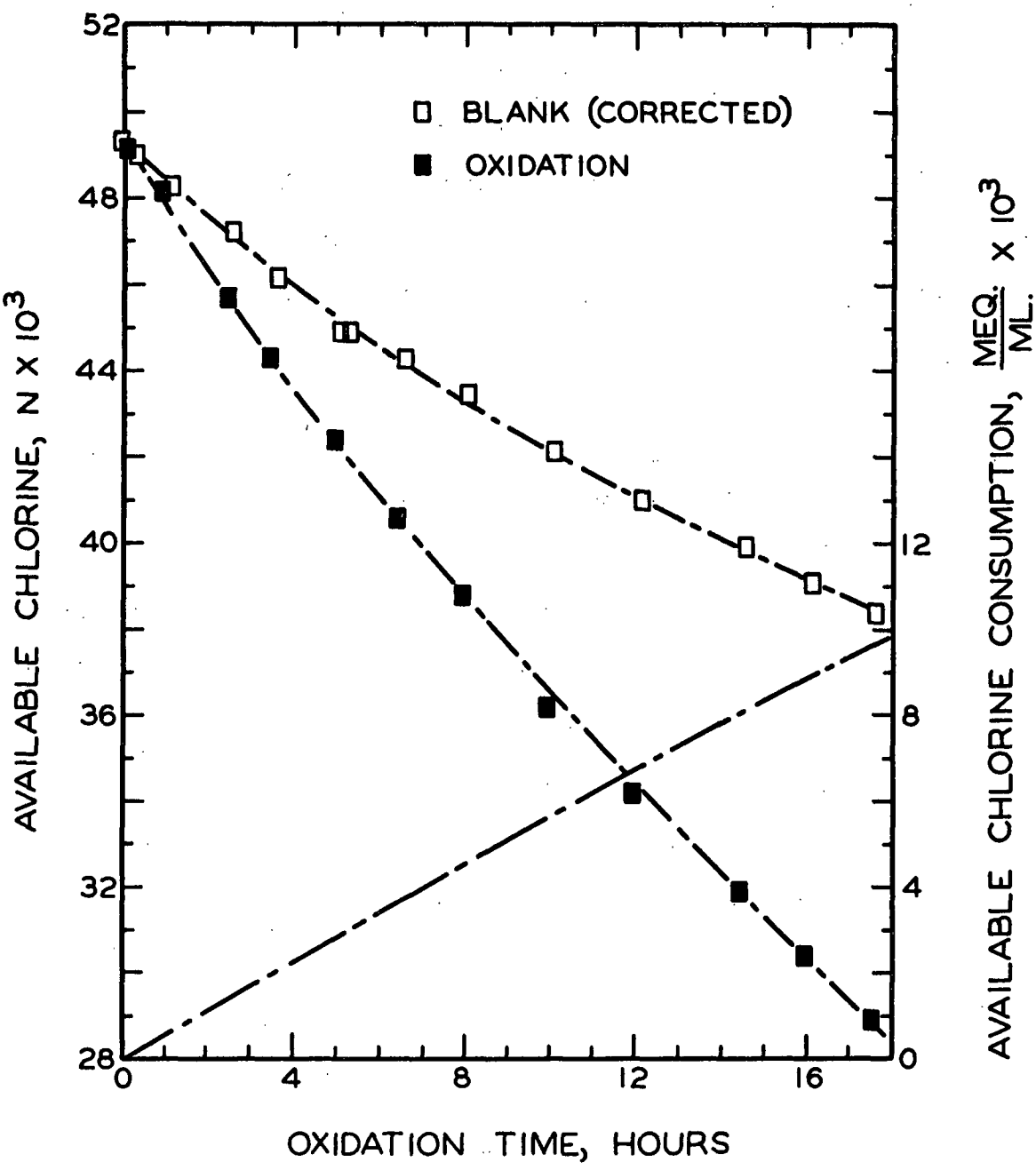

Figure 7. Concentration and Consumption of Available Chlorine During the Oxidation of Methyl β-D-glucopyranoside(aglucone-^{14}C) (Oxidation 8)

OXIDATIONS

EQUIPMENT

The apparatus used for the oxidations, Fig. 8, consisted of a vapor-tight, opaque, three-neck reaction flask equipped with a magnetic stirring bar, a combination electrode for pH measurements, an entrance for sodium hydroxide solution for pH control, a serum-cap entrance for inserting the needle of a syringe, a gas inlet, and a gas outlet. The reaction flask was immersed in a 25°C. constant-temperature bath. The oxidation solution was automatically kept at pH 7 by the addition of sodium hydroxide solution through a solenoid valve[1] which was controlled by auxiliary circuitry to a modified Beckman model H-2 pH meter.

Connecting the auxiliary circuitry into the pH meter removed the electric current from the pH meter dial and transferred it to a special dial equipped with a manual pointer and a microswitch.[2] As the pH of the solution dropped, the pH pointer swung past the manual setting on the special dial. This caused the microswitch and the auxiliary circuitry to open the solenoid valve, thereby adding sodium hydroxide solution from the buret. As the pH of the solution increased, the solenoid valve was closed automatically in a similar manner. This automatic control of pH was accurate to $\pm$ 0.15 pH units during the three main oxidations.

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[1] A continuous-duty solenoid valve designed for use as the delivery valve on the Sargent-Malmstadt Automatic Spectro/Electro Titrator, Model-SE, E. H. Sargent Co., Chicago, Illinois.

[2] Available from Assembly Products Inc., Chesterland, Ohio.

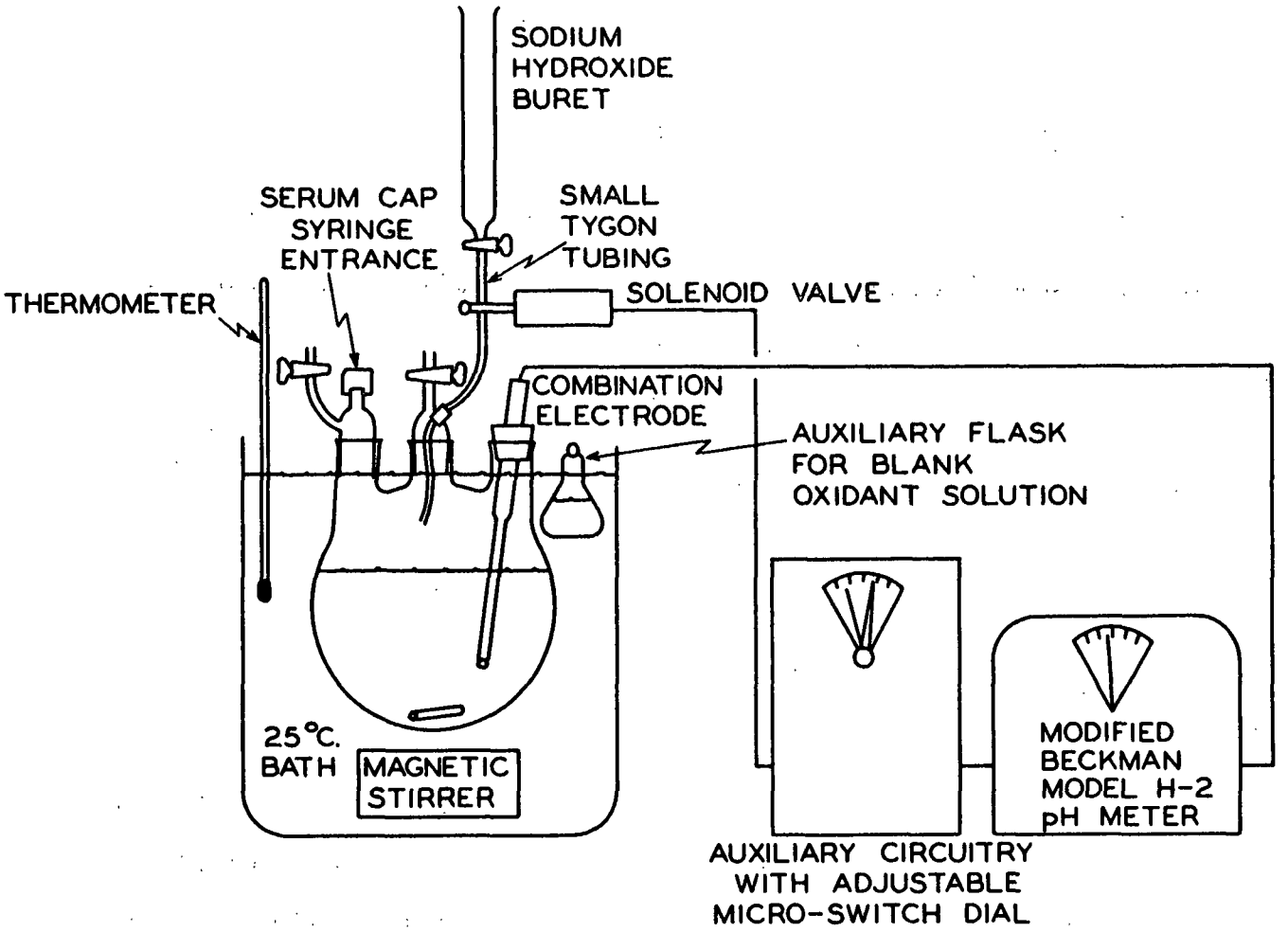

Figure 8. Oxidation Equipment

PRELIMINARY OXIDATIONS

Eight oxidations were made in this study. The first five were preliminary oxidations in which more was learned about the system and how to control it. The details about these oxidations will be discussed in Appendix V. Oxidation seven, while not a preliminary oxidation, will also be discussed in Appendix V.

OXIDATION[1] OF METHYL β-D-GLUCOPYRANOSIDE(GLUCOSYL-^{14}C)

Preparation of Methyl β-D-Glucopyranoside(glucosyl-^{14}C)

Glucose[2], uniformly labeled with carbon-14, was used to synthesize the methyl β-D-glucopyranoside(glucosyl-^{14}C), hereafter referred to as MBG*, used in oxidation six. The 2.8 mg. of glucose(^{14}C) was diluted with 2.5 g. of ordinary glucose and dissolved in 25 ml. of 50% ethanol. Fifteen milliliters of this solution containing 1.5 g. of glucose was further diluted with 13.5 g. of ordinary glucose to give diluted glucose(^{14}C), with a calculated activity of 22,200 disintegrations per minute per milligram of carbon, dis./(min.)(mg. C).

Crystals were not obtained from this solution of glucose(^{14}C). To obtain anhydrous material, the glucose was dissolved in an acetic anhydride-acetic acid mixture. The solution was concentrated to a thick sirup. This concentration in the presence of acetic anhydride was repeated several times. Then the glucose(^{14}C) was converted to acetobromoglucose(^{14}C) in acetic anhydride solution by the method described on page 16. The yield was 35.3 g. of thick sirup.

The acetobromoglucose(^{14}C) was converted to methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside(^{14}C), or MTABG*, according to the method described on page 17. Two crops of crystals and a sirup were obtained. Table II shows data on this yield.

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[1] Oxidation six.

[2] One-tenth millicurie of crystalline D-glucose(^{14}C), uniformly labeled, lot 2128, produced biosynthetically by 10 to 12-hour photosynthesis of detached *Canna indica* leaves in the presence of carbon dioxide(^{14}C), was purchased from Volk Radiochemical Co., Chicago, Illinois. The company specified that the 2.8 mg. of glucose had a specific activity of 6.3 mc./mM. and was 98% pure by carrier dilution analysis as the pentaacetate.

TABLE II

YIELD OF METHYL β-GLUCOSIDE TETRAACETATE(GLUCOSYL-^{14}C)

Crop	State	Weight, g.	Melting Point[a], °C.	Yield, %
1	Crystals	12.490	103.0-105.5	41.4
2	Crystals	0.999	97-99	--
3	Thick sirup	14.14	--	--

[a]The literature value is 104-105°C. (29).

These crystals and the sirup were deacetylated according to the method on page 18 to give MBG*. Crystalline MBG* was obtained from the deacetylation of the first crop of MTABG*. This MBG* was characterized and recrystallized. The recrystallized product was also characterized. Table III gives data on these products. Recrystallization did not change the specific activity. Although some glucose impurity remained, the recrystallized product was used in preliminary Oxidation 5.

After recrystallizations, all crops of MBG* were combined and purified according to the method given on page 19. The purified MBG* was characterized, and that data is also included in Table III. The specific activity was unchanged by purification. This purified MBG* was used in Oxidation 6, one of the two duplicate oxidations in this study.

Oxidation

An aqueous chlorine solution was prepared as described on page 20 and allowed to equilibrate 40 hours. Available chlorine concentration data taken during this equilibration are recorded in Appendix IX, and shown in Fig. 4. After equilibration, a sample of this oxidant was transferred to a separate flask to serve as a blank.

TABLE III

PROPERTIES OF METHYL β-GLUCOSIDE(GLUCOSYL-^{14}C)

Product	Weight, g.	Melting Point,[a] °C.	Rotation,[b] $[\alpha]_D$ (c 2, water)	Sp. Activity[c]	Chromatographic Impurities
Crude[d]	4.164	105.5-108.5	-32.5°	15,760	Glucose & 1 unknown
Recrystd.	2.484	107.0-109.5	-34.0°	15,720	≤ 1% Glucose
Purified[e]	4.802	107.0-107.5[f]	-34.0°	15,800	< 0.02%[g]
Unlabeled[h]	--	107.0-109.5	-34.0°	--	< 0.1%

[a] Literature values vary from 104 to 116°C.

[b] Calculated on the basis of anhydrous MBG (weight reduced by 4.43% to account for hemihydrate water). Literature values vary from -32 to -34°.

[c] Disintegrations per minute per milligram of glucosyl carbon.

[d] From the first crop of MTABG*.

[e] Purified by treating with alkali after combination of all impure MBG*, including the recrystallized material which still contained some glucose.

[f] Softening started at 106.5°C. Several fine crystals on the sides of the tube melted at 112.5°C.

[g] Pure in three solvent systems: A, B, and C, Appendix III.

[h] Included in table for comparison.

A water solution of 0.9758 g. of MBG* was then injected by syringe into the reaction flask. Periodically, samples of both the blank solution and the oxidation solution were withdrawn for determination of the available chlorine content. The data collected are recorded in Appendix IX, which also shows the corrected data for the blank solution. This correction takes into account the dilution in the oxidation solution, by the MBG* solution and the pH control sodium hydroxide solution, which does not occur in the blank solution. Figure 5 shows the results.

A 100-ml. sample of the oxidation solution was removed after nine hours' oxidation time. Sulfurous acid was added to this sample to destroy the remaining available chlorine, thus stopping the oxidation. The pH was readjusted to approximately 7 with normal sodium hydroxide, and the solution was stored in a refrigerator. The remainder of the solution was allowed to react for 18 hours, and then the oxidation was stopped by similar injections into the oxidation flask.

Calculations[1] were made to find the solids content, especially the carbohydrate content, of the oxidation solutions (Table IV). Then the calculated weight of carbohydrates, in the aliquots of the oxidation solutions removed for analysis, was used to convert the yields of products to percentages. For an aliquot of the 18-hour oxidation solution, the calculated solids content using the data in Table IV was 796 mg. The experimental result found when the aliquot was concentrated to dryness was 780 mg., a 2% difference. The oxidation solutions were analyzed for various products and for unreacted MBG*. The first analysis was for carbon dioxide.

TABLE IV

CALCULATED SOLIDS CONTENT OF OXIDATION SOLUTIONS

Solid	9-Hr. Solution,[a] grams	mg./ml.	18-Hr. Solution,[b] grams	mg./ml.
NaCl	1.69	15.68	5.80	15.50
$NaClO_3$	1.31	12.15	4.75	12.69
Na_2SO_4	0.27	2.50	0.75	2.00
Carbohydrates[c]	0.1944	1.803	0.6082	1.625

[a]Total volume: 107.8 ml.

[b]Total volume: 374.3 ml. Volume of gaseous phase: 702 ml.

[c]Calculated as unreacted MBG*.

[1]An example of these calculations is shown in Appendix IX.

Analysis

Carbon Dioxide

Ten per cent of both the liquid and the gaseous phases of the 18-hr. oxidation solution was transferred into the apparatus used in carbon dioxide analysis. The apparatus and technique are described in detail in Appendix VI. Essentially, the technique consisted of sweeping all carbon dioxide(^{14}C) from a sample and absorbing it in a sodium hydroxide solution. An aliquot of the sodium hydroxide solution was then transferred into the Van Slyke manometric apparatus where the carbon dioxide was again liberated. The carbon dioxide was then transferred into a Bernstein-Ballentine counting tube where the radioactive count was determined. The sweeping-out with nitrogen was continued until the count reached a constant value, thus indicating all the carbon dioxide had been removed from the sample. From this count, the amount of carbon dioxide product in the oxidation solution was calculated. Of course, this method determined only the carbon dioxide originating in the labeled portion of the glucoside.

The first attempt at analysis for this oxidation was unsuccessful because the carbon dioxide was removed too slowly from the sample. In a second trial, also using 10% of both phases from the oxidation flask, this difficulty was corrected by proper pH adjustment in the sample while sweeping out the carbon dioxide. This pH adjustment was incorporated into the method as described in Appendix VI.

In this second trial, five counts were made after 19 to 40 hours of nitrogen flow to sweep out the carbon dioxide. No increase in activity was observed after 19 hours. Calculations showed 1.99 $\pm$ 0.04% of the glucosyl carbon[1] was oxidized

[1]Percentages are based on the total amount of starting material.

to carbon dioxide in 18 hours in the aqueous chlorine. Data and calculations are given in Appendix VI, page 104-5.

Methanol

Although it was not expected that any significant amount of methanol(^{14}C) would be produced in the oxidation of MBG*, the analysis for this product was carried out. An isotope dilution technique was desirable here, as it was in the other analyses in this study. Because volatile compounds would be lost during the evacuation of the Van Slyke apparatus before the wet combustion to carbon dioxide, it was necessary to form some solid derivative from the methanol. The solid derivative formed in this study was methyl p-nitrobenzoate.

In the analysis, the isotope dilution step was made first, before any separations or reactions. A known amount of methanol was added to an aliquot of the oxidation solution. Methanol was then separated by distillation, and reacted with p-nitrobenzoyl chloride to give methyl p-nitrobenzoate (methyl-^{14}C). Details of this procedure are given in Appendix VI, page 106-9 . The specific activity of the ester was then determined using the Van Slyke apparatus and a Bernstein-Ballentine counting tube. From a corrected isotope dilution factor[1] and the specific activity, the amount of methanol in the oxidation solution could be calculated.

[1]Dilution of the radioactive methanol from the glucosyl portion of the MBG* could come from both the methanol added for isotope dilution and from the unlabeled aglucone methanol split off of the MBG* during the oxidation. The latter amount was unknown until an analysis had been made for methanol in oxidation seven in which M*BG was oxidized. Using two identical oxidations, one with MBG* and one with M*BG, the methanol formed in both runs could be calculated, Appendix VI.

In this oxidation the results were obtained (page 108) by two methods. In the first method 0.11 ± 0.06% of the glucosyl carbon appeared as methanol when calculated by using only the dilution of the methanol added to the solution. In the second method 0.14 ± 0.08% of the glucosyl carbon appeared as methanol when calculated by using the corrected dilution. The activity of the samples was so low that the 90% confidence limits on the activity were about half as large as the activities themselves. However, the important point is that the amount of methanol obtained from the glucosyl portion of MBG during aqueous chlorine oxidation is very small, as would be expected.

Unreacted Glucoside

The solid content of the oxidation solution was about 95% inorganic salt and 5% carbohydrate. Preliminary attempts at isotope dilution analysis showed that these salts would have to be removed before crystalline, isotopically diluted MBG could be isolated. After diluting an aliquot of the oxidation solution with unlabeled MBG, three methods were used to remove the salts. These methods were: extraction of the carbohydrates from the dried oxidation solution solids with n-propanol, chromatographic purification using heavy chromatographic paper, and crystallization. The exact order of use varied during this study. Appendix VI gives the details of each of these three techniques and summarizes the exact order in each of the isotope dilution analyses.

After isolating pure crystalline MBG from the dilution samples, the specific activity was determined using the methods of APPENDIX II. The amount of unreacted glucoside was then calculated. The results are shown in Table XXV, page 115. The averages for duplicate results in Oxidation 6 showed 87.55% of the starting MBG remained after 9 hours' oxidation, and 84.8% remained after 18 hours'

oxidation. The amount of MBG oxidized was therefore 12.45% at 9 hours and 15.2% at 18 hours.

Glucose

To a 25-ml. aliquot of the 18-hour oxidation solution, 153 mg. of unlabeled glucose was added. The solution was concentrated to dryness. The carbohydrates were extracted with n-propanol and the extract chromatographically purified according to the procedures given in Appendix VI. Solvent A of Appendix III was used in the chromatographic purification.

The solution extracted from the chromatograms was concentrated to dryness under reduced pressure. The solids, 70 mg. of sodium acetate, and 0.35 ml. of acetic anhydride were sealed in an ampule and heated in a boiling water bath for two hours. Addition of 1.7 ml. of water gave crude glucose pentaacetate crystals. Three recrystallizations from ethanol were used to purify the pentaacetate. The melting point was 131.0-131.5°C. The literature value is 135°C. (33).

The specific activity of the pentaacetate was 8.9 ± 2.2 dis./(min.)(mg. total C) and the percentage of glucose in the carbohydrates of the oxidation solution was calculated to be 0.57%.

Arabinose

Arabinose formed during the 18-hour oxidation of MBG* was determined by using the same isotope dilution techniques as were used in determining unreacted MBG*. Procedure "D" of Appendix VI, Table XXVI was used. Duplicate results showed that 0.78 and 1.26% of the carbohydrate material in the oxidation solution was arabinose. The average was 1.02%.

The accuracy of this result depends upon how completely the arabinose was extracted with n-propanol, because the isotope dilution was not the first step in procedure "D". This is probably the reason for the poor agreement between the duplicate samples. Any such error would cause a low result.

OXIDATION[1] OF METHYL β-D-GLUCOPYRANOSIDE(AGLUCONE-^{14}C)

Preparation of Methyl β-D-Glucopyranoside(aglucone-^{14}C)

Equimolar amounts, 0.0495 mole, of methanol[2] labeled with carbon-14 and unlabeled acetobromoglucose[3] were used in the preparation of the M*BG by the method which begins on page 15. Two crystalline crops and a sirup of the intermediate product, M*TABG, were obtained. Table V shows the yields and melting points.

TABLE V

YIELD OF METHYL β-D-GLUCOSIDE TETRAACETATE(AGLUCONE-^{14}C)

Crop	State	Weight, g.	Melting Point[a], °C.	Yield, %
1	Crystals	12.151	103.5-105.0	67.8
2	Crystals	0.842	99.5-101.0	4.7
3	Sirup	4.96[b]	--	--

[a]The literature value is 104-105°C. (29).

[b]Maximum M*TABG content, calculated from the theoretical yield.

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[1]Oxidation eight.

[2]One-half millicurie of methanol(^{14}C), lot 2500D, containing 1.0 mc./mM. of carbon-14 was purchased from Volk Radiochemical Co., Chicago, Ill. The original 16 mg. of methanol(^{14}C) was diluted with pure unlabeled methanol to a volume of 5 ml., 3.9713 g., to give a calculated activity of 7.45 x 10^5 dis./(min.)(mg. C).

[3]The acetobromoglucose used had a melting point of 88.5-90.0°C. and $[\alpha]_D$ = +195.3° (c 2, chloroform). The corresponding literature values are 88-89°C. (27), and +197.8° (27).

The crystals and sirup were deacetylated. Chromatography showed the deacetylated sirup contained only a small amount of M*BG. It was not further worked up.

After purification by the method on page 19, 5.739 g. of crystalline M*BG was obtained. It had a melting point of 108.5-111.5°C. with some prior softening at 106°C. This was the highest melting point for MBG obtained in this study. The rotation $[\alpha]_D = -34.5°$ (c 2, water) was the most negative optical rotation for MBG obtained in this study. The literature values are given in Table III. The M*BG was chromatographically pure in developers A, B, and C of Appendix III. Because very small amounts of glucose would have been found if present, and because glucose is the most probable impurity, the $(M*BG)_2 \cdot H_2O$ was estimated to be at least 99.95% pure.

The specific activity of the M*BG was found to be 788,200 dis./(min.)(mg. aglucone C). This was 106% of the activity calculated from the dilution of the original methanol(^{14}C).

Oxidation

The fresh aqueous chlorine oxidant was prepared as previously described, page 20, to be identical with that used in the oxidation of MBG*. More oxidant was prepared, because this oxidation was scaled up by a factor of three. During equilibration, the loss of available chlorine was slightly more rapid than in the equilibration for the oxidation of MBG*. Therefore, instead of allowing the equilibration to proceed the full 40 hours, the M*BG was injected after 32 hours and 50 minutes. The concentration of available chlorine was then the same for the two oxidations. The available chlorine concentration data taken during the equilibrations are recorded in Appendix IX and shown in Fig. 4. Just before

injection of the M*BG, a sample of the oxidant was transferred to a separate opaque flask to serve as a blank.

A water solution of 2.9306 g. of M*BG was injected. The starting conditions for this oxidation were identical with those for the oxidation of MBG* in Oxidation 6, Table XIII. Available chlorine concentration was followed in both the oxidation and the blank solution. The data are recorded in Appendix IX and the results are shown in Fig. 7. Figure 15 shows that the change in available chlorine concentration in this oxidation of M*BG, Oxidation 8, was very nearly identical with the change during the oxidation of MBG*, Oxidation 6.

Samples of both the liquid and gaseous phases were removed with a syringe after 1.5, 4, and 9 hours' oxidation. These samples were transferred into opaque, evacuated flasks of known volume. Sulfurous acid was added to destroy the remaining available chlorine, and sodium hydroxide solution was added to readjust the pH to about 7. These solutions were also added with a syringe[1], which also was used to withdraw small samples to test for the complete destruction of the available chlorine[2], and to find the pH. The sample flasks, Fig. 9, were then stored in a refrigerator.

After 18 hours, the oxidation in the main flask was similarly stopped, the pH adjusted, and the flask stored in a refrigerator. Table VI shows the calculated carbohydrate content of these solutions. This calculated amount of carbohydrates was later used to convert the yields of products to percentages.

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[1]The needle of the syringe was long enough to reach through the stopcock to the bottom of the sample flasks.

[2]Starch-potassium iodide test paper was used.

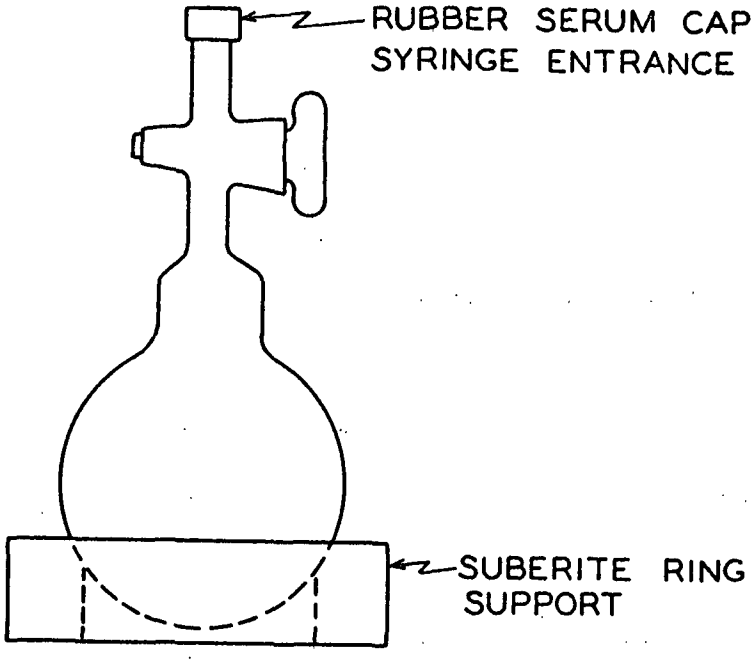

Figure 9. Oxidation Solution Sample Flasks

TABLE VI

CALCULATED CARBOHYDRATE CONTENT OF OXIDATION SOLUTIONS

Oxidation Time, hr.	Volume, ml. Liquid	Vapor	Carbohydrates,[a] mg.	mg./ml.
1.5	294.4	215.4	488.4	1.659
4	297.6	222.4	485.9	1.633
9	190.8	316.6	349.8	1.833
18	730.2	1478.0	1349.0	1.848

[a]Calculated as unreacted M*BG.

The four oxidation solutions were analyzed for various products and for unreacted M*BG.

Analysis

Carbon Dioxide

Fifteen per cent of the 1.5, 4, and 9-hour oxidation solutions and vapors and 5% of the 18-hour solution and vapor were analyzed for carbon dioxide by the method of Appendix VI. Table VII shows the results. Appendix IX gives the data, and an example of the calculations is given in Appendix VI.

TABLE VII

PERCENTAGE OF AGLUCONE CARBON FOUND AS CARBON DIOXIDE

Oxidation Time, hr.	Carbon Dioxide Found, %
1.5	0.0028 ± 0.0020
4	0.020 ± 0.003
9	0.024 ± 0.004
18	0.089 ± 0.004

Methanol

Duplicate 20-ml. samples of the oxidation solutions were taken for isotope dilution analyses for methanol by the method given in Appendix VI. Results from the previous oxidation (glucosyl-labeled glucoside) showed very little methanol was produced from the glucosyl group. Therefore, it was not necessary to correct for traces of methanol from that source when making isotope dilution calculations. After calculating the specific activity of the methanol from the specific activity found in the methyl p-nitrobenzoate, normal isotope dilution calculations (Equation 3, page 13) gave the results in Table VIII.

TABLE VIII

PERCENTAGE OF AGLUCONE CARBON FOUND AS METHANOL

Oxidation Time, hr.	Methanol Found, % (Duplicate Samples)
1.5	1.82 ± 0.04, 1.91 ± 0.04
4	2.51 ± 0.05, 2.63 ± 0.05
9	4.90 ± 0.03, 4.68 ± 0.05
18	8.52 ± 0.07, 8.67 ± 0.09

Unreacted Glucoside

The method for these analyses is given in Appendix VI where the results are given in Table XXV. Only one acceptable result for the amount of unreacted M*BG was obtained for the 1.5-hr. sample. That result, and the averages of four acceptable results for the other samples, is given in Table IX.

TABLE IX

RESULTS OF ANALYSIS FOR UNREACTED GLUCOSIDE

Oxidation Time, hr.	Unreacted M*BG, %	M*BG Oxidized, %
1.5	95.6	4.4
4	91.6	8.4
9	85.6	14.4
18	77.6	22.4

Formaldehyde and Formic Acid

Duplicate analyses for each oxidation solution were made to determine formaldehyde and formic acid. The method given in Appendix VI was used. The percentage of aglucone carbon oxidized to formaldehyde and formic acid is shown in Table X.

TABLE X

PERCENTAGE OF AGLUCONE CARBON FOUND AS FORMALDEHYDE AND FORMIC ACID

Oxidation Time, hr.	Formaldehyde, % (Duplicate Analyses)		Formic Acid, % (Duplicate Analyses)	
1.5	0.146 ± 0.005,	0.148 ± 0.005	0.031 ± 0.003,	0.022 ± 0.003
4	0.248 ± 0.006,	0.262 ± 0.005	0.040 ± 0.003,	0.053 ± 0.003
9	0.513 ± 0.004,	0.523 ± 0.006	0.082 ± 0.003,	0.079 ± 0.003
18	1.024 ± 0.010,	1.064 ± 0.010	0.121 ± 0.004,	0.161 ± 0.003

Hydrolyzable Methanol

The aglucone carbon from the oxidized M*BG may be present in the oxidation solutions as unreacted M*BG, carbon dioxide, methanol, formaldehyde, formic acid, or as a methoxyl group still attached to some oxidized residue from the glucosyl group[1]. The analyses for the first five of these products have been discussed and the results presented. The aglucone carbon in the compounds of the last category listed above was determined in two steps: first an hydrolysis, and then a determination of the methanol produced. From the result of this methanol determination, the free methanol previously found and the amount of methanol hydrolyzed from the unreacted M*BG were subtracted. The method is described in Appendix VI.

The results from this hydrolysis method were disappointing. Table XI and Fig. 10 show that the results obtained were quite scattered. A least-squares line was calculated for these data; it is

[1]The ketoglucosides would be in this category; other glucosyl residues could be diacids and dialdehydes.

$$P = 92.43 - (0.1829)(H) \qquad (4),$$

where P is the percentage of aglucone carbon found as methanol after hydrolysis, and H is the oxidation time in hours. According to this equation, only 92.43% of the aglucone carbon was hydrolyzable at zero reaction time. Of course this is not correct, but it is interesting that during tests of the method[1], using solutions with the same concentrations in M*BG and salts as in the oxidation solutions at zero reaction time, the average result was 92.25%. These two results are so nearly the same that there is some logic in dividing Equation (4) by 0.9243, so that at zero time the amount of hydrolyzable methanol is 100%, as it should be. The corrected equation is, then,

$$P = 100 - (0.1979)(H) \qquad (5).$$

Table XI shows the corrected results using this equation.

TABLE XI

RESULTS OF HYDROLYZABLE METHANOL DETERMINATIONS

Oxidation time, hours	1.5	4	9	18
Hydrolyzable methanol, %[a]	88.63	93.19	88.45	89.68
(Original results)	93.32	93.42	90.48	89.41
Corrected hydrolyzable methanol[b], %	99.70	99.21	98.22	96.44
Methanol on glucosyl residue[c], %	2.23	5.04	7.83	10.24
Total aglucone carbon found[d], %	99.88	99.53	98.84	97.71
Aglucone carbon unaccounted for, %	0.12	0.47	1.16	2.29

[a]Per cent, in this table, is the per cent of the aglucone carbon in the starting glucoside which was found in the given product.

[b]Using Equation (5).

[c]These results are obtained by subtracting the free methanol (Table XII) and the methanol that would have been formed by hydrolysis of the unreacted M*BG (Table XII) from the corrected hydrolyzable methanol.

[d]These results are obtained by adding the corrected hydrolyzable methanol and the formaldehyde, formic acid, and carbon dioxide results shown in Table XII.

[1]These tests are discussed in Appendix VI, pages 121-5.

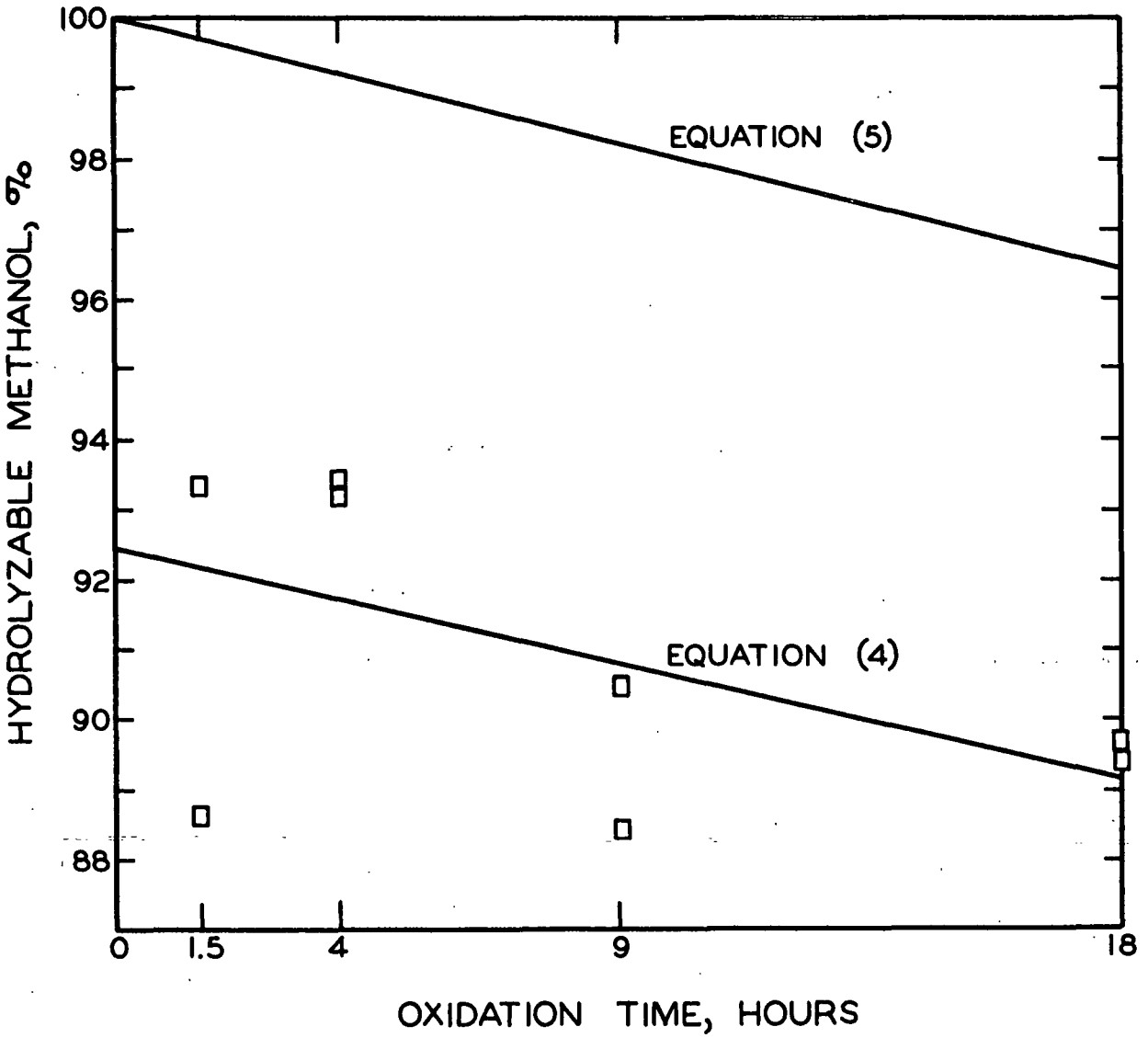

Figure 10. Per Cent of Aglucone Carbon Found as Hydrolyzable Methanol

Subtracting the amount of free methanol and the amount of methanol hydrolyzed from unreacted M*BG, the amount of methanol still bound to an oxidized glucosyl residue was calculated. Also, by adding the aglucone carbon found as carbon dioxide, formaldehyde, formic acid, and in this analysis the percentages of the aglucone accounted for were calculated. Table XI also shows this result.

DISCUSSION

ANALYTICAL RESULTS

A summary of the analytical results from this study appears in Table XII. Most of these data are also shown in Fig. 11, 12, and 13, where the different data are easily compared.

The glucosyl portion of the MBG has been shown to be the source of over 95% of the carbon dioxide oxidation product. The amount of carbon dioxide produced from the aglucone group is very small. These new data further explain the source of the carbon dioxide found by Henderson (8), who could only suggest that it came, in part, from the Carbon 1 position or the aglucone carbon.

The methanol reaction product from the aglucone group does not account for more than 30 to 45% of the aglucone carbon from the oxidized MBG. This, together with the comparatively low yields of formaldehyde, formic acid, and carbon dioxide, suggests that much methanol must remain attached to an oxidized glucosyl residue. Oxoglucosides could account for part of the remaining aglucone carbon. In fact, in a somewhat similar oxidation, Theander (7) did find about a 5% production of these oxoglucosides, and in this study an attempt to analyze for these compounds as a group gave a result of 10.2%, although the method was not too successful (p. 42). Other such products could be diacids and dialdehydes with methoxyl groups.

The above results, and Daniel's conclusion (12) (that Carbon 1 can be the source of not more, and probably less, than 21% of the saponifiable carbon dioxide from cellulose oxidized by aqueous chlorine at pH 4.5), give further evidence that the oxidative attack is not primarily centered at the Carbon 1-glycosidic oxygen-carbon portion of the molecule, but is centered, to a large extent, in that

TABLE XII

SUMMARY OF THE ANALYTICAL RESULTS[a]

Starting material	MBG*[b]		M*BG[b]				M*BG	
Oxidation number	6		8				7	
Oxidation time, hr.	9	18	1.5	4	9	18	9	18
Methanol[c]	--	0.14	1.87	2.57	4.79	8.60	4.15	6.28
Formaldehyde[c]	--	--	0.147	0.255	0.518	1.044	--	--
Formic acid[c]	--	--	0.027	0.047	0.081	0.141	--	--
Carbon dioxide[c]	--	1.99	0.003	0.020	0.024	0.089	0.011	0.037
Glucose[d]	--	0.57	--	--	--	--	--	--
Arabinose[d]	--	1.02	--	--	--	--	--	--
Methanol on glucosyl residue[c,e]	--	--	2.23	5.04	7.83	10.24	--	--
MBG Unreacted, %	87.6	84.8	95.6	91.6	85.6	77.6	84.2	82.5
MBG Oxidized, %	12.4	15.2	4.4	8.4	14.4	22.4	15.8	17.5

[a]Most of these results are averages. For the individual results see the experimental techniques and results section (Oxidations 6 and 8) or Appendix V (Oxidation 7).

[b]Duplicate oxidations except for the position of the label.

[c]Per cent of glucosyl (Oxidation 6) or aglucone (Oxidations 7 and 8) carbon found as this compound.

[d]Per cent of starting MBG*.

[e]A correction factor had to be used to obtain these results. See p. 43.

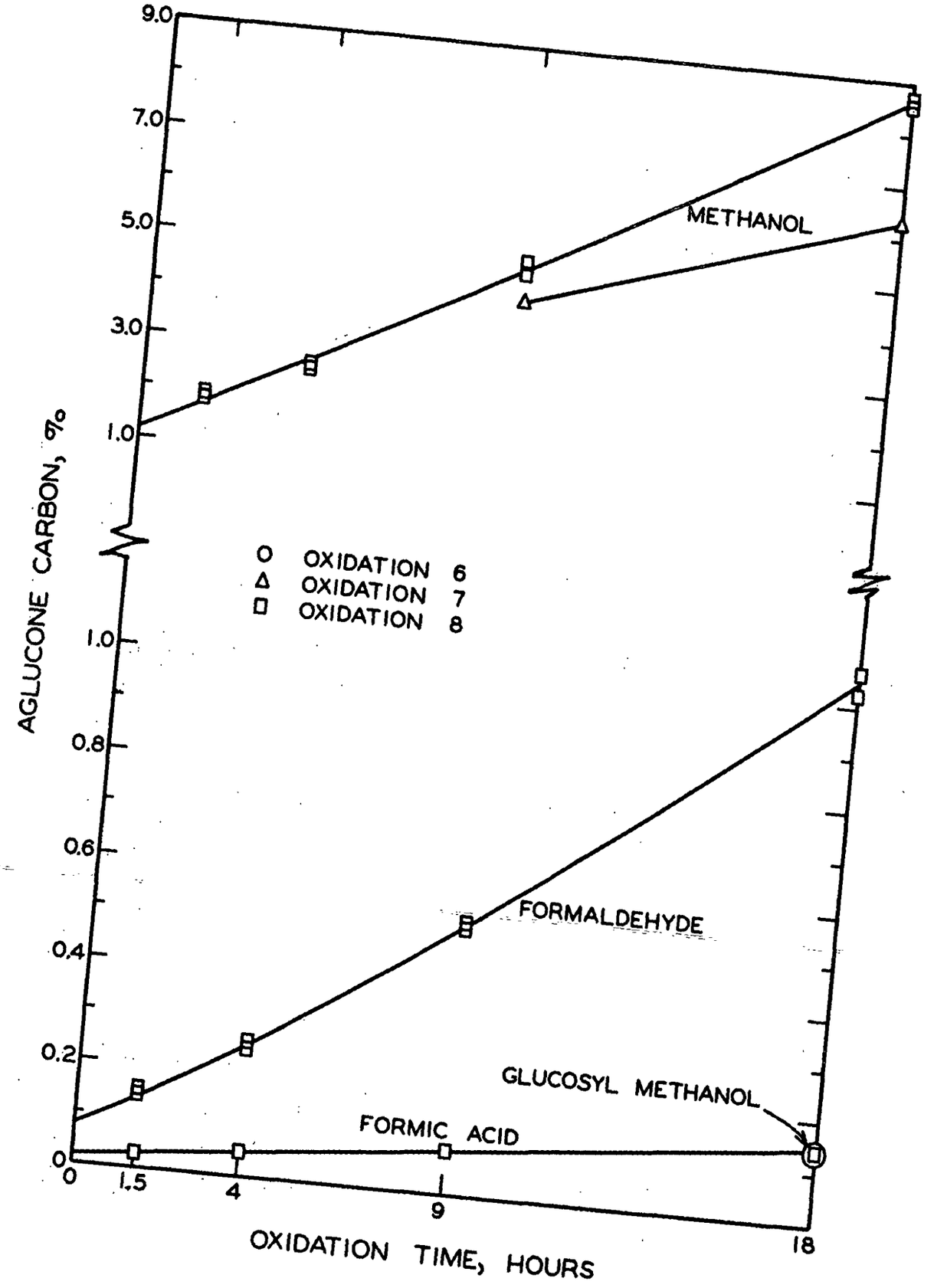

Figure 11. Per Cent of Aglucone Carbon Found as Methanol, Formaldehyde, and Formic Acid (Note the Change in Scale)

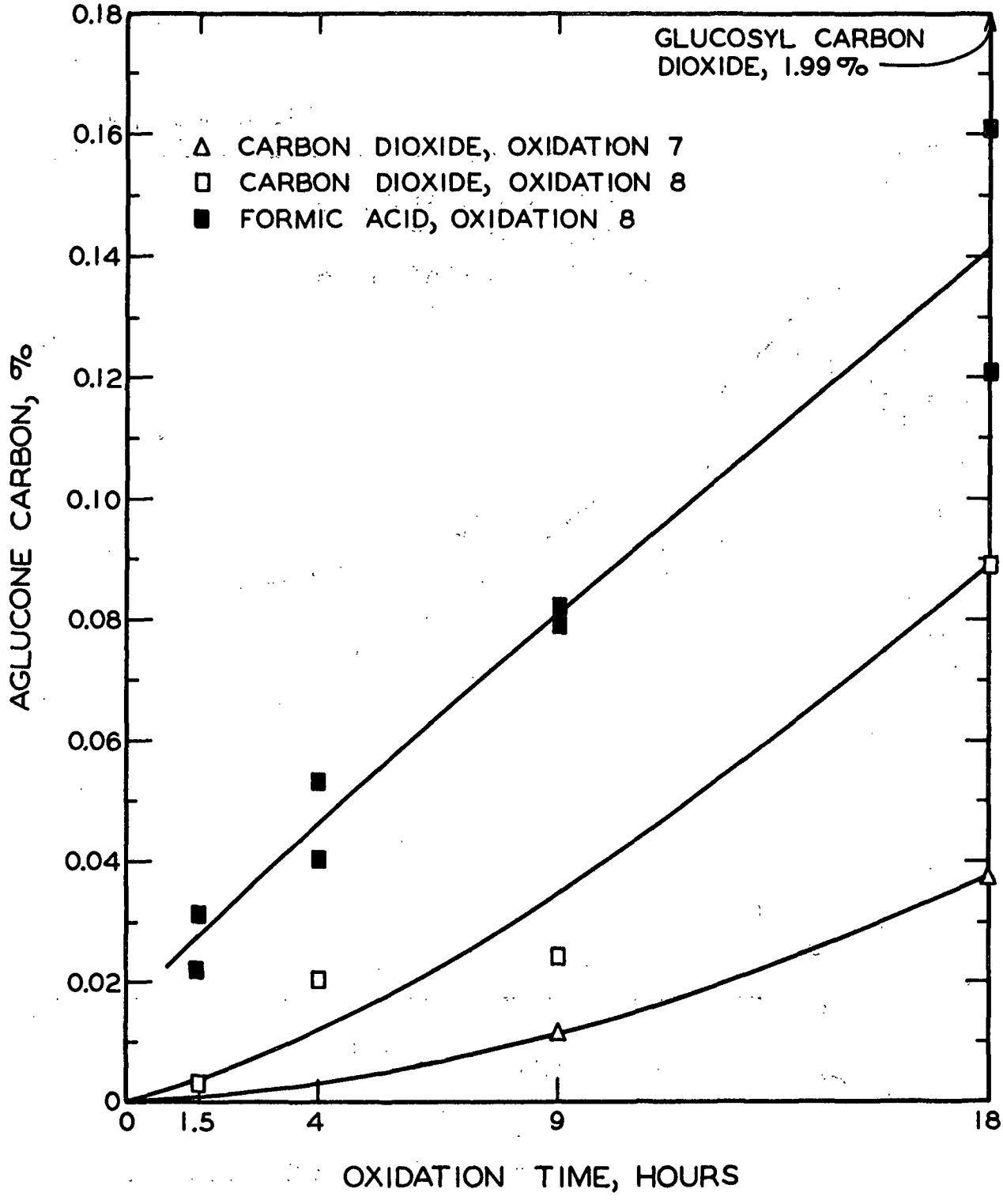

Figure 12. Per Cent of Aglucone Carbon Found as Formic Acid and Carbon Dioxide

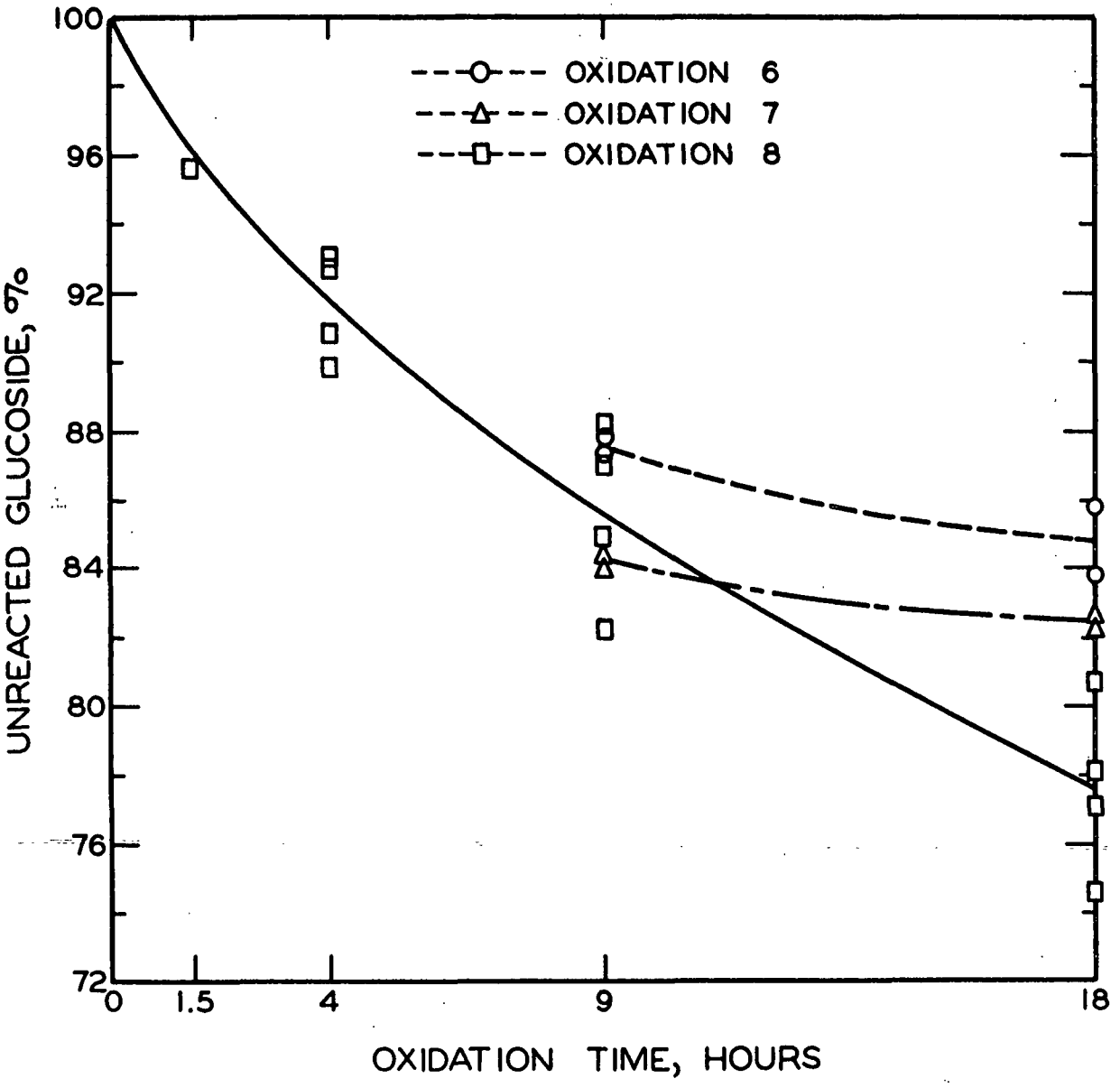

Figure 13. Per Cent Unreacted Glucoside

portion of the glucosyl group not directly involved in the glycosidic linkage. This is similar to the conclusion of Whistler and co-workers (14, 16), that the oxidative attack tends to be specific for the carbon two and carbon three positions, especially in the vicinity of pH 7.

Possible pathways by which various reaction products are formed are shown in Fig. 14. Step A would be the result of an oxidative attack at Carbon 1; Step B would be an attack at carbon atoms 2,3,4,5, or 6; and Step C would be an attack on the aglucone carbon. Step D is an hydrolysis, and the remaining steps are oxidations.

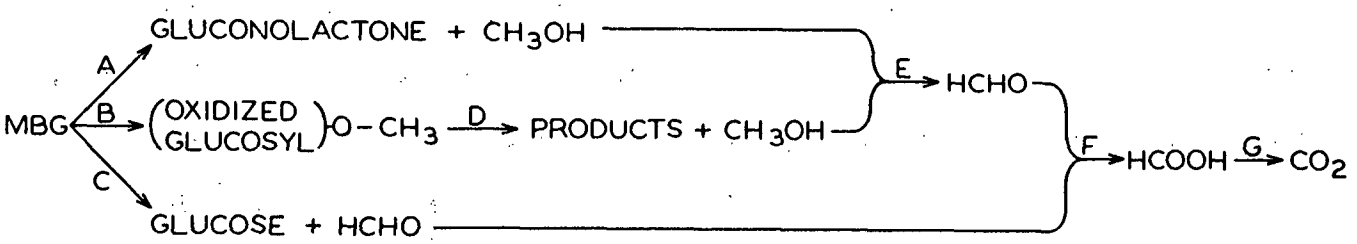

Figure 14. Possible Paths for the Reactions

Assuming all the carbon dioxide(^{14}C) found in the oxidation of M*BG came from formic acid by Step G, which in turn came from formaldehyde by Step F, a corrected curve for the formaldehyde produced could be drawn. Such a curve would have a positive slope which increases with the time of oxidation, just as the formaldehyde curve and carbon dioxide curves do in Fig. 11 and 12 (the formic acid curve is very nearly a straight line). This means that the rate of formaldehyde production increased during the oxidation. If it had been produced only by oxidation of the aglucone group of M*BG, Step C above, the rate would have decreased because the concentration of M*BG was decreasing during the oxidation. Therefore, there must have been other paths, such as A + E and B + D + E, by which it was produced. Nevertheless, finding some glucose (the arabinose-to-

glucose ratio in this study was approximately two to one, which confirms Henderson's ratio) as product shows that either the aglucone was undergoing some oxidation by Reaction C above, or that there was hydrolysis. Workers in this field have agreed that oxidation is not preceded by hydrolysis (6, 8, 10).

Reactions E, F, and G are all secondary oxidations and increase the oxidant consumption. Correcting for this secondary oxidant consumption leaves about 4.2 meq./mM instead of 4.4 meq./mM to be applied to the glucosyl portion of the M*BG. Strictly speaking, only 2 meq. of oxidant was consumed for each millimole of M*BG oxidized, because after the first oxidative attack the material is no longer M*BG. Therefore, it may be concluded that, on the average, each millimole of primary product consumed an additional 2.2 meq. of oxidant in this study. This, and the fact that the concentration of primary products never approached the concentration of the M*BG, shows that some or all of the primary oxidation products from the glucosyl group are more easily oxidized than the starting material.

One might expect that this would also be true for the products from the aglucone group. In support of this idea, it has been found that the acetaldehyde produced in the oxidation of ethanol with bromine is in turn oxidized to acetic acid, with the secondary oxidation increasing in rate until finally the primary and secondary oxidations proceed at the same rate, with the concentration of acetaldehyde becoming constant (34). The data show that such a rapid secondary oxidation did not occur in this study for products from the aglucone group; the formaldehyde is not rapidly oxidized to formic acid. In fact, in the methanol to formaldehyde to formic acid to carbon dioxide series, the rates of oxidation apparently decrease. The ratio of formaldehyde to methanol remained nearly constant at approximately one to ten, increasing slightly during the oxidation.

Similarly, the ratio of formic acid to formaldehyde remained nearly constant at about one to six, decreasing slightly during the oxidation. This decrease was probably due to loss of formic acid through oxidation to carbon dioxide.

In Fig. 11 and 12, the methanol, formaldehyde, and formic acid curves do not extrapolate back to zero at zero oxidation time. For formic acid and formaldehyde this may be experimental error. However, it has been reported elsewhere that such a methanol curve extrapolated back to about one per cent at zero time when methyl α-D-glucopyranoside was oxidized with hypochlorous acid (35). A possible explanation for the behavior of the methanol curve would be that an isotope effect or other experimental error was occurring in the analysis procedure. In a test of the method (page 110), the results were high at the lowest methanol concentration, and low at the middle and highest methanol concentrations. Whether this was a consistent experimental error or not is not known. The isotope effect is improbable. Elaborate fractionating columns would be needed to significantly fractionate labeled and unlabeled methanol. For example, the difference in the boiling point of unlabeled methanol and of methanol(^{13}C) is 0.0055°C. (36). In the reaction to form methyl p-nitrobenzoate, the carbon atom of the methanol is not directly involved in the reaction center. A significant isotope effect in this step is therefore unlikely. If the analyses are correct, methanol must be produced rapidly for a short time at the start of the reaction. No satisfactory explanation for this is known.

In the hydrolysis technique discussed on page 42, only about 92% of the MBG was hydrolyzed under conditions that should have given very nearly 100% hydrolysis. Enough unlabeled methanol for isotope dilution had been added at the start of the hydrolysis to dilute the methanol from the MBG about 56 times. This excess of methanol in the solution could have caused the incomplete hydrolysis. A

reverse reaction,

$$CH_3OH + \text{Glucose} \longrightarrow \text{MBG} \qquad (6),$$

could have been detected by isolating some of the MBG remaining at the end of the hydrolysis and redetermining its specific activity. Reverse reaction would have made the specific activity lower than that of the starting material because of all the unlabeled methanol in the solution. These hydrolysis solutions had a very high salt concentration, and isolating enough of the final MBG for activity determination was not accomplished.

The results after 18 hours in Oxidation 8 are an example of the type of aglucone-carbon balance obtained in this study. After 18 hours' oxidation, when 22.4% of the M*BG had been oxidized, 8.60% of the aglucone carbon was found as free methanol, and 10.2% as methanol formed by hydrolysis of oxidation products; it must be remembered that a correction factor had to be used to obtain this latter figure (p. 43). After adding the amount of aglucone carbon found as formaldehyde, formic acid, and carbon dioxide, 2.29% of the aglucone carbon was still unaccounted for. The cumulative experimental errors in such a summative analysis preclude any attempt to account for the remaining 2.29% without additional experimental results. Other products from the aglucone group are possible in this system, such as methyl chloride[1], methyl formate[2], or glucosyl and oxidized glucosyl residues with an oxidized aglucone group still attached. These were considered less probable products than the ones determined in this study.

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[1] In the oxidation of ethanol with bromine, no bromination was found (34), but iodoform has been found when aldehydes are oxidized with alkaline hypoiodite (37). Also, the mechanism proposed by Lichtin and Saxe would indicate such a product (19).

[2] Some ethyl acetate has been found as a product of the bromine oxidation of ethanol (34).

MECHANISM AND ISOTOPE EFFECT

The starting conditions for the oxidations are compared in Table XIII, and the concentration of available chlorine during the oxidations is shown in Fig. 15. The intention was to have two identical oxidations, one labeled in the glucosyl group and the other labeled in the aglucone group. This was accomplished in Oxidations 6 and 8. The oxidant concentration, glucoside concentration, and therefore the oxidant-to-glucoside ratio, are the same for the two oxidations. The concentration of oxidant decreased in very nearly the same manner during the two oxidations, and at the end of the oxidation equal amounts of oxidant had been consumed by the glucoside.

The oxidation conditions and total oxidant consumption were the same for the two oxidations, but the analyses for unreacted glucoside showed more oxidation for the aglucone-labeled glucoside than for the glucosyl-labeled glucoside. Therefore, calculations using this data showed that 6.5 meq. of oxidant had been consumed for each millimole of glucosyl-labeled glucoside oxidized, while the consumption for the aglucone-labeled glucoside was only 4.4 meq./mM. While the analysis for unreacted glucoside did show some scatter (Fig. 13), it is felt that there is a significant difference in the results for the two oxidations. Yet, it is difficult to see, in light of the identical starting conditions and oxidant consumptions during the oxidations, how 15.2% of the MBG* and 22.4% of the M*BG could have been oxidized (Table XIII).

It is very interesting that Oxidation 7 using M*BG, which had starting conditions similar but not identical to those of the above oxidations, also had an oxidant consumption of 4.4 meq./mM. This would indicate that the difference in the analyses could be attributed, at least in part, to the position of the carbon-14 label.

TABLE XIII

COMPARISON OF OXIDATIONS, STARTING CONDITIONS AND RESULTS

Starting material		MBG*[a]	M*BG[a]	M*BG
Oxidation		6	8	7
Starting conditions				
Available chlorine,[b]	N	0.0491	0.0493	0.0466
Solution volume,[b]	ml.	500	1500	491.2
Oxidant,	meq.	24.55	73.95	22.89
Glucoside,[b]	g.	0.9758	2.9306	0.9206
	mM	5.025	15.092	4.740
Total meq. oxidant/mM MBG		4.89	4.90	4.83
Total mM MBG/ml.		0.01005	0.01006	0.00965
Results				
Loss of available chlorine due to oxidation, meq./ml.[c]		0.0099	0.0098	0.0075
MBG oxidized in 18 hr., %		15.2[d]	22.4[e]	17.5[f]
mM MBG oxidized/ml.		0.00153	0.00225	0.00169
meq. oxidant consumed by MBG / mM MBG oxidized		6.5	4.4	4.4

[a]Underlining indicates duplicate oxidations.

[b]Appendix IX.

[c]Figures 5, 6, and 7.

[d]Page 35.

[e]Table IX.

[f]Page 100.

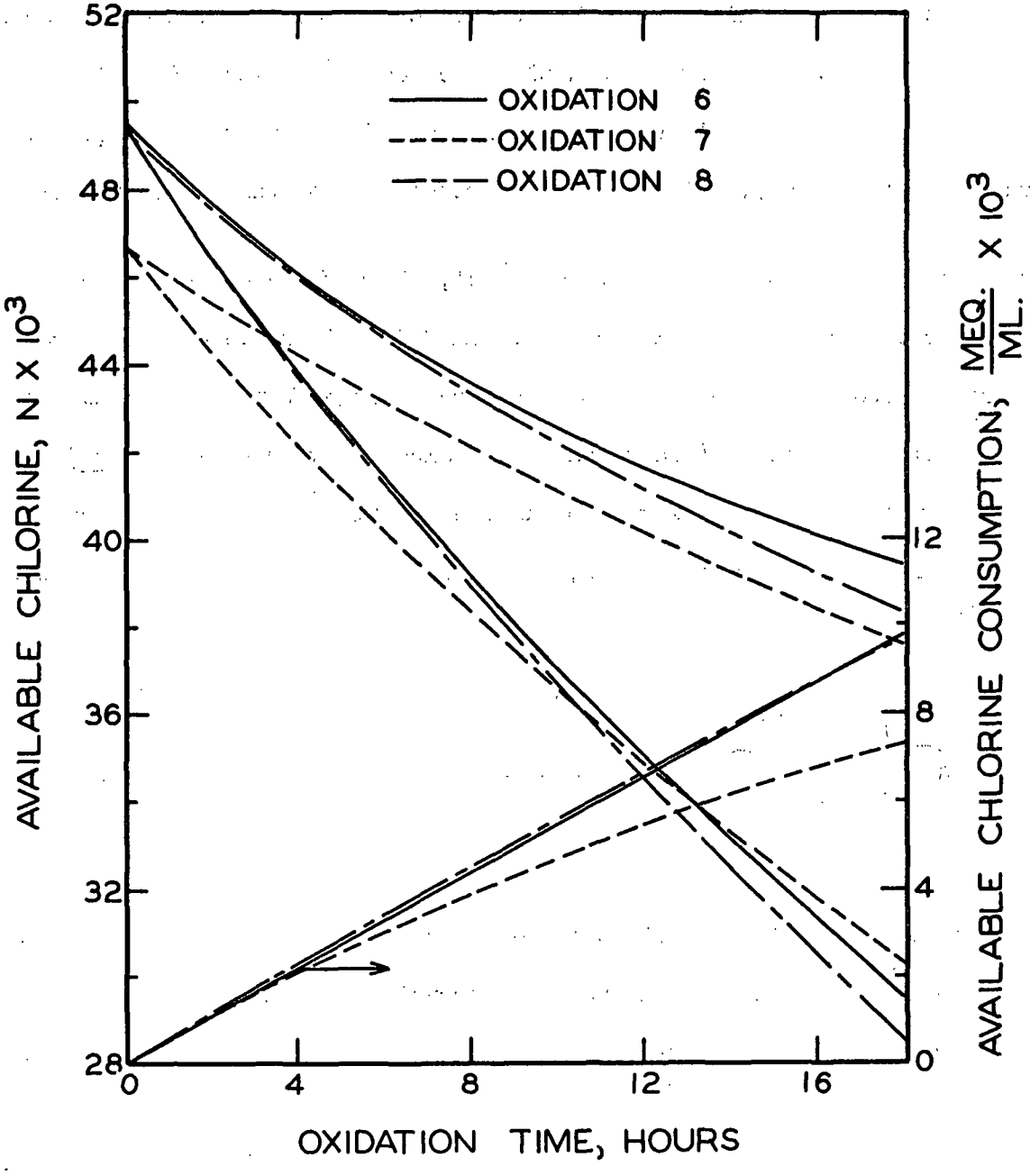
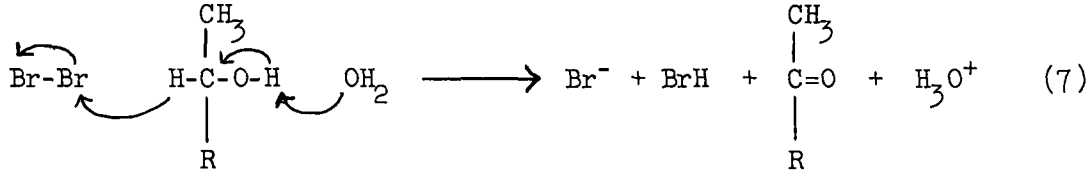


Figure 15. Concentration and Consumption of Available Chlorine During the Oxidation of Methyl β-D-gluco-pyranoside (Comparison of Oxidations 6, 7, and 8)

Therefore, it may be that an isotope effect was causing the results of the analyses for the unreacted glucoside to be different. In the introduction it was concluded that the error due to the isotope effect would not be greater than 10%. Assuming, for the moment, that it was 10%, and that it caused low results for the amount of MBG* oxidized (high results for the amount of MBG* unreacted), the correct amount of MBG* oxidized would have been 23.7% instead of 15.2%, and the oxidant consumption would have been 4.2 meq./mM. instead of 6.5 meq./mM. Within experimental accuracy, this is the same result as for the other oxidations.

To have a large (primary) isotope effect, the carbon-14 atoms have to be involved directly in the reaction center. In MBG* this would mean the breaking of a carbon-carbon, carbon-oxygen, or a carbon-hydrogen bond. There has been work in similar oxidations (the oxidation of alcohols with bromine) (20), which showed that the rate-determining step was the breaking of a carbon-hydrogen bond by hydride transfer from carbon, followed by fast proton removal from the oxygen.



(7)

In oxidation of glucose with bromine, a hydride transfer was also suggested (38). While hydride transfer has not been proved in the oxidation of glucosides, and this mechanism does not agree with the ideas of all workers[1], it is quite

[1]Some of these ideas were mentioned briefly in the introduction.

possibly the actual mechanism[1]. If hydride transfer is the mechanism, then a carbon-hydrogen isotope effect would be expected. Hine (40) lists the maximum for this isotope effect as 4.1% (Table XIV). The 10% isotope effect assumed above would then be too large, and more of the disagreement in the values for the oxidant consumption would have been experimental error.

Then, there was another assumption, because the above discussion would imply that the hydride transfer from each of the six carbon atoms in the glucosyl group was equally probable. While there is evidence that the attack can occur at any carbon atom, there is also evidence that the attack tends to be specific at Carbon 2 and Carbon 3. The oxoglucosides with carbonyl groups at positions 2,3,4, and 6 have been obtained as products from glucoside oxidations (7), which shows that there is some random attack. However, in the oxidation of starch components and of methyl 4-O-methyl-β-D-glucopyranoside (14, 16), the attack tended to be specific for positions two and three, especially at pH 7.

There are 63 possible labeled species in randomly labeled glucose; however, the probability for any molecule to contain two or more carbon-14 atoms is extremely small in the glucoside used in this work and all that need be considered are the six singly labeled species labeled on positions one through six. The chance for a labeled molecule to be labeled on position two or three is therefore one third. The error due to the isotope effect is therefore not as large for oxidation of uniformly labeled glucosyl groups when the attack is specific for

[1]In the studies of the bromine oxidation of alcohols, the hydride was transferred to the $Br_{2(aq)}$ molecule. There was very little $Cl_{2(aq)}$ present in the oxidant solutions in this study, and a comparison of this work with Grillo's work (discussed later) shows much of the oxidation was hypochlorite ion-catalyzed oxidation by hypochlorous acid. Of course, the detailed mechanism for such an oxidation is not known, and could possibly be some sort of hydride transfer also.

positions two and three as it is for random attack. For example, two molecules, one labeled in position six and the other unlabeled, would react at the same rate, and there would be no isotope effect for these two molecular species. The error due to the isotope effect would then have a maximum of one third of the 4.1 or 1.4%. Then, even more of the difference in the values for oxidant consumption would have to be experimental error.

In summary of the above discussion, the results of the oxidant consumption calculations suggest that an isotope effect was being detected, but the effect cannot be used for mechanism studies because the specificity of the attack on the uniformly labeled glucosyl group is not known, and the experimental error masked the effect.

KINETICS

The rate of oxidant consumption in this study at 25°C. was calculated from the initial slopes of the oxidant consumption curves in Fig. 5, 6, and 7, and found to be

$$-\frac{d(Ox)}{dt} = 3.24(10^{-4})(MBG)(Ox) \qquad (8),$$

where the units are moles/liter and seconds.

This result was compared with one found by using calculations of the type used by Grillo (9), his rate and catalytic constants for 35.7°C., and the initial concentrations in this study. The resulting equation was

$$-\frac{d(Ox)}{dt} = 16.8(10^{-4})(MBG)(Ox) \qquad (9).$$

The ratio of the constants in the two equations is 5.2 to 1.0, while a ratio of about two to one would have been expected because of the temperature difference. Considering the assumptions necessary in making the calculations, particularly regarding the activity coefficients, the agreement between the two studies is quite good. The detailed calculations for this comparison are shown in Appendix VII, in which it may be seen that hypochlorite ion-catalyzed oxidation by hypochlorous acid was, according to these calculations, the major factor in the oxidations.

CONCLUSIONS

Investigation of the products produced from the aglucone group when carbon-14 labeled methyl β-D-glucopyranoside is oxidized with aqueous chlorine at 25°C. and pH 7 has shown that the main oxidative attack is on the glucosyl unit. Only 5.7% of the aglucone carbon from the glucoside which had been oxidized was accounted for as the aglucone oxidation products: formaldehyde, which contained 4.7%, formic acid, which contained 0.6%, and carbon dioxide, which contained 0.4% of this aglucone carbon. The attack on the glucosyl portion of the glucoside was accompanied by the release of methanol into the solution, which was found to account for 38.4% of the aglucone carbon from the oxidized glucoside. Most of the remaining aglucone carbon was found by hydrolyzing the reaction products and redetermining the methanol in solution. This showed that most of the remaining aglucone carbon must have remained on an oxidized glucosyl residue, and thus, much of the oxidative attack is not on the Carbon 1-aglucone portion of the glucoside, but is on the remainder of the glucosyl group.

The methyl group is the site of some oxidative attack while it is still the aglucone group of the glucoside, but this is not the only oxidation which it undergoes. It is also converted to formaldehyde, either from free methanol in solution, or from a methoxyl (or an oxidized methoxyl) group on some of the primary reaction products.

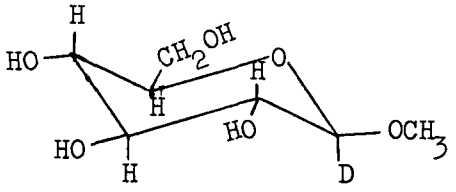
Subsequent oxidation of formaldehyde to formic acid does not occur nearly as rapidly as the oxidation of acetaldehyde to acetic acid in bromine oxidations. There is appreciable oxidation of formic acid to carbon dioxide, although in the over-all system much more carbon dioxide has its origin in the glucosyl unit than in the aglucone group. Under the conditions used in this study, this ratio was over twenty to one.

Another important conclusion, especially for anyone studying these aqueous chlorine oxidations in neutral or nearly neutral systems, is that a very careful account must be taken of the disproportionation of the available chlorine oxidant into chlorate and chloride, neither of which oxidize the glucoside, if an accurate determination of the oxidant consumption by the glucoside is to be made.

SUGGESTIONS FOR FUTURE RESEARCH

An oxidation of methanol(^{14}C) in the aqueous chlorine system used in this study, followed by analysis for formaldehyde, formic acid, and carbon dioxide, would show what portion of the methanol was oxidized as an aglucone group and what portion was oxidized after becoming free methanol in solution. Methyl chloride and methyl formate should be included in the analysis scheme. Then, another oxidation of aglucone-labeled glucoside followed by analysis for methyl formate and methyl chloride would help elucidate the mechanism of the oxidations.

Isotope effects could be studied much better using specifically labeled glucoside. Particularly, glucoside labeled in the Number 1 and in the Number 2 positions should be studied. Carbon-14 is useful, but when an isotope effect is being studied, deuterium-labeled glucoside such as



would give a bigger effect if a carbon-hydrogen bond is being broken. Such an isotope effect would help explain the mechanism of the oxidation.

Ideally, work with a better model compound for cellulose, such as methyl β-cellotrioside, with only the central glucosyl unit labeled with carbon-14 would give results more directly relatable to cellulose oxidations. However, starting material production and oxidation product identification would be very difficult tasks.

GLOSSARY

ABG — 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide

ABG* — 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide(glucosyl-^{14}C)

MBG — Methyl β-D-glucopyranoside

MBG* — Methyl β-D-glucopyranoside(glucosyl-^{14}C)

M*BG — Methyl β-D-glucopyranoside(aglucone-^{14}C)

MTABG — Methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside

MTABG* — Methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside(glucosyl-^{14}C)

M*TABG — Methyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside(aglucone-^{14}C)

dis./(min.)(mg.C)
Disintegrations per minute per milligram of total carbon

dis./(min.)(mg. glucosyl C)
Disintegrations per minute per milligram of glucosyl carbon

dis./(min.)(mg. aglucone C)
Disintegrations per minute per milligram of aglucone carbon

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APPENDIX I

ISOTOPE EFFECT

An isotope effect, a difference in the rates of reaction of labeled and unlabeled molecules, is of two categories. When the isotope is directly involved in the rate-determining step of a reaction, a relatively large difference (primary isotope effect) is found. When the isotope is not directly involved, but is suitably located with respect to the reaction center, there may be smaller effects (secondary isotope effects). An isotopic atom remote from the reaction center has little or no effect on the reaction rate (39).

The difference in nuclear mass of the normal and the isotopic atom causes a difference in the energy of activation for a reaction. This contribution to the isotope effect is less at higher temperatures because more molecules are occupying higher energy levels. Nuclear mass also affects the breaking of bonds in the activated complex, but this effect is independent of temperature (24). Hine (40) has listed some approximate maximum values for this kinetic isotope effect (Table XIV). It will be noticed that the effect is much smaller for carbon isotopes where the ratio of the weights is small than it is for the hydrogen isotopes where the weight ratio is two or three.

TABLE XIV

APPROXIMATE MAXIMUM VALUES FOR THE KINETIC ISOTOPE EFFECT (40)

Bond		Temp. °C.	k/k^*
C-H	C-D	25	6.9
C-H	C-T	25	16.0
C^{12}-H	C^{14}-H	25	1.041
C^{12}-C^{12}	C^{12}-C^{14}	25	1.092
C-O^{16}	C-O^{18}	25	1.063

Hine states that in spite of the approximations which must be made to obtain such maximum values, no values of $\log(k/k^*)$ more than about 25% higher than those listed in the table have been established[1], and a number of isotope effects around the size of those listed have been reported.

One system which has been well studied in relation to the isotope effect is the dehydration of formic acid with sulfuric acid. In one case carbon-14 was used (41), and in another case carbon-13 was used (42). Table XV shows these isotope effects.

TABLE XV

ISOTOPE EFFECTS IN THE DEHYDRATION OF FORMIC ACID

Temp. °C.	k^{12}/k^{14}[a]
0	1.126
14.75	1.101
18.75	1.098
24.75	1.094
	k^{12}/k^{13}[b]
-30	1.0799
-26	1.0767
-20	1.0753
-15	1.0720
0	1.0664

[a] Data of Ropp and co-workers (41).

[b] From the data of Bigeleisen and co-workers (42).

[1] The $\log(k/k^*)$ term is written here as Hine gives it, even though k/k^* is used in the table. $\text{Log}(k/k^*)$ is probably correct. Increasing k/k^* by 25% when its value is 1.041 would give 1.301, thus increasing the isotope effect from 4 to 30%. Increasing $\log(k/k^*)$ by 25% would mean increasing k/k^* from 1.041 to 1.052, thus increasing the isotope effect from 4 to 5%. This latter range is much more reasonable for carbon isotope effects.

It is possible to calculate the carbon-14 isotope effect if the carbon-13 isotope effect is known (42). Doing this, the above results can be put on a common basis. Figure 16 is the result of this calculation and shows that isotope effects decrease with increasing temperature.

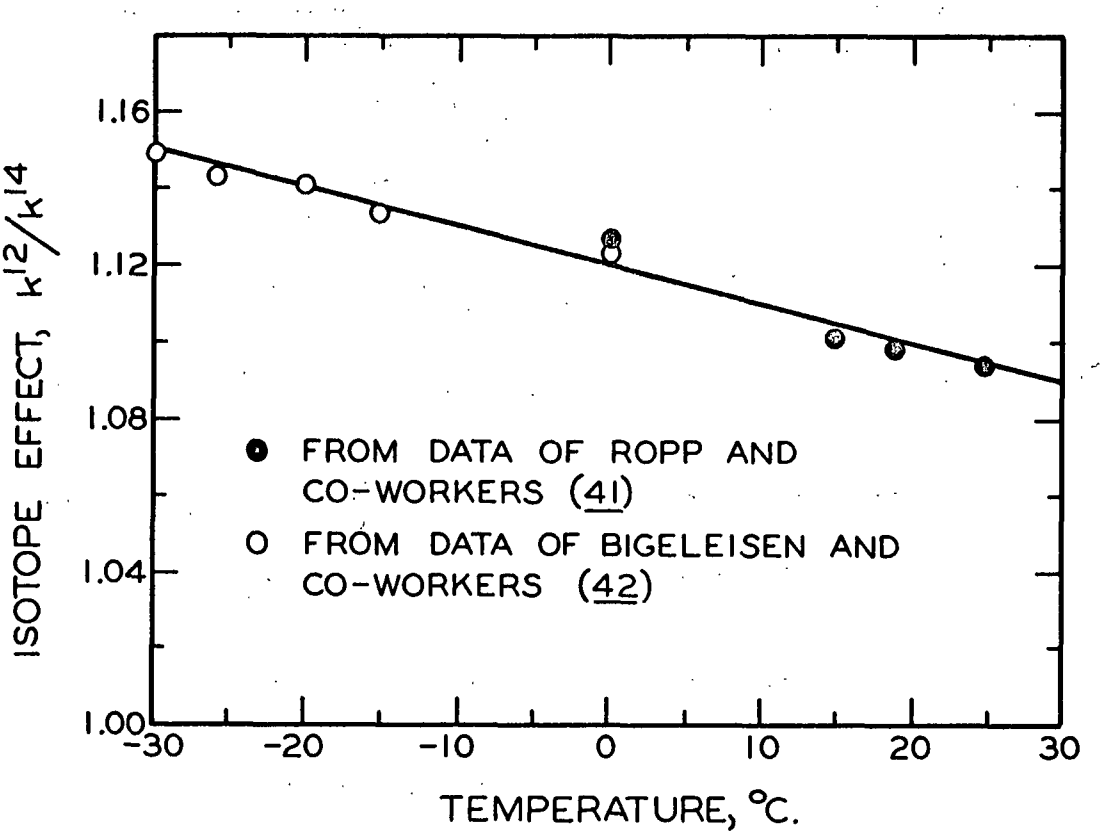

Figure 16. Decrease of Isotope Effect with Temperature

When a reaction is complete, there is no error due to isotope effects, because all molecules of both isotopic species have reacted. If the isotope effect is very large, as in the case of hydrogen and deuterium, the relative amounts of the two species reacted is very different until the reaction is very nearly complete. This could lead to a large error in tracer experiments unless the effect was taken into account. In the case of carbon isotopes, the rates are not far different for the two species, and the error is less (43). When carbon-14 is used in tracer experiments, the amount of error caused by the

different rates of reaction for the two isotopic species can be calculated for any given per cent completion of the reaction, if the relative rates are known. Assuming k/k^* values of 1.10 and 1.05, this error has been calculated and is shown in Fig. 17.

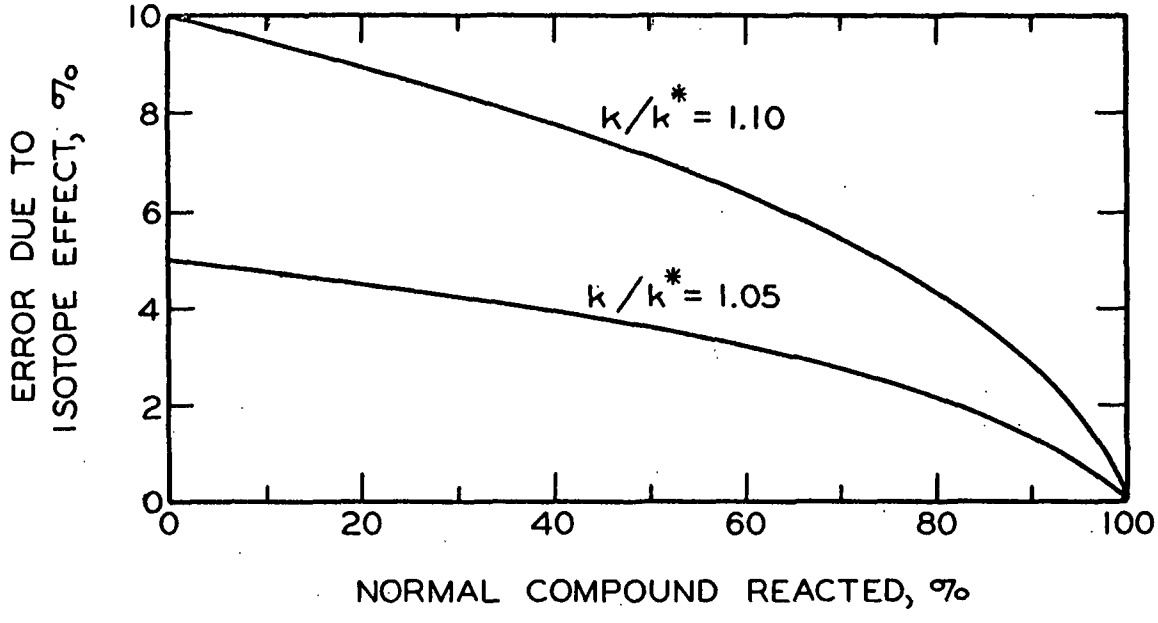

Figure 17. Error Due to the Isotope Effect During a Reaction

The isotope effect for carbon-14 compounds during wet combustion in the Van Slyke apparatus has been shown for several compounds to be in the range zero to four per cent (44). Since the reaction in this apparatus is 99 to 100% complete, the error due to the isotope effect in this step of the analyses in this study is very nearly zero.

In the main reaction in this study, the oxidation of MBG was approximately 20% complete. If there was a 10% isotope effect, the error due to the effect would have been about 9% (Fig. 17). If the rate-determining step was the breaking of a carbon-hydrogen bond, as has been shown for similar systems (20, 38), then the isotope effect would have been about 4% (Table XIV), and the error because of it would have been correspondingly lower.

Probably the safest conclusion to draw is that the error in this study due to isotope effects was not larger than 10%. This is the same conclusion that Daniel made about his work (12).

APPENDIX II

MEASUREMENT OF SPECIFIC ACTIVITY

THOMAS-VAN SLYKE MANOMETRIC APPARATUS

The Van Slyke apparatus, Fig. 18, is a device for converting organic materials to carbon dioxide and giving a quantitative measure of the amount of carbon in the sample. The amount of carbon is calculated from pressure, volume, and temperature measurements on the carbon dioxide.

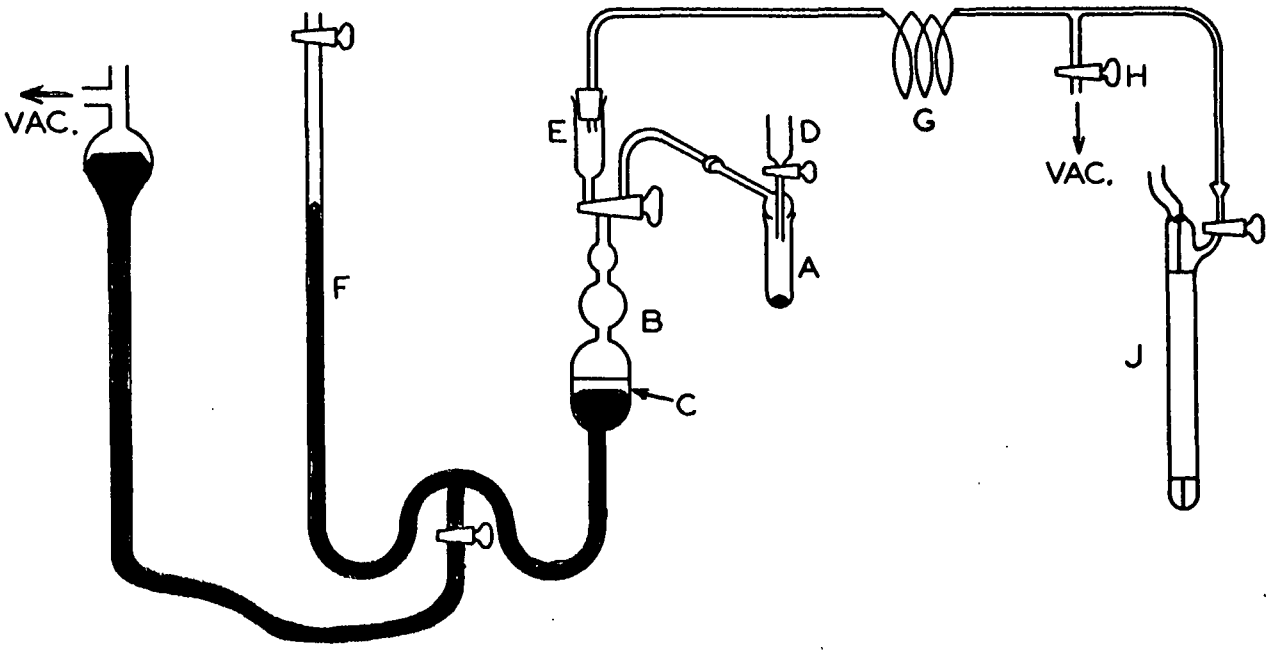

Figure 18. Thomas-Van Slyke Manometric Apparatus and Bernstein-Ballentine Tube

The wet combustion reagents used in the apparatus are potassium iodate, potassium dichromate, phosphoric acid, and either concentrated or fuming sulfuric acid, depending on the sample to be burned (45).

Van Slyke and Folch published their original description of the technique in 1940 (46). Other workers have also described the details of the method (12,

47). With the advent of the widespread use of carbon-14, an article describing how the apparatus could be used in conjunction with gas-phase counters for activity measurements was published by Van Slyke, Steele, and Plazin (48). Then, in 1962, Van Slyke and Plazin explained in another article how to transfer the carbon dioxide from the manometric apparatus into the counting tube without the use of liquid nitrogen (49).

The methods given in the above articles were used in this study. With these articles available, only a brief description of the procedure will be repeated here.

Combustion tube A, Fig. 18, containing the dry weighed sample and the dry combustion reagents, is attached to the apparatus. The combustion tube and the connecting tubing are evacuated using the mercury in the gas buret, B, as a piston. Sodium hydroxide-hydrazine solution is placed on the mercury surface, C. The acid combustion reagent is added to the sample from reservoir D. Heat is applied which oxidizes the sample to carbon dioxide. This carbon dioxide is absorbed in the sodium hydroxide-hydrazine solution. All remaining air is expelled from gas buret B. The carbon dioxide is then released by adding lactic acid from reservoir E. The volume of the carbon dioxide is adjusted with the mercury. The pressure is measured using manometer F, and the temperature is measured in the water jacket surrounding the gas buret. After correcting for water vapor and a blank, factors supplied by Van Slyke and Folch (46), are used to calculate the milligrams of carbon in the sample.

BERNSTEIN-BALLENTINE PROPORTIONAL COUNTING TUBES

The radioactive carbon dioxide in the Van Slyke apparatus is now transferred into the Bernstein-Ballentine counting tube for measurement of the activity. The method used in this study did not require the use of liquid nitrogen (49).

The counting tube, J, Fig. 18, and the tubing connecting it with reservoir E are evacuated by vacuum pump through stopcock H. The carbon dioxide is then passed through reservoir E and freezing coils G which remove water vapor, into the counting tube. Gas buret B is then filled with methane which is used as a piston to push the last traces of carbon dioxide into the counting tube. The counting tube is removed from the Van Slyke apparatus and filled to atmospheric pressure with methane; it is now ready for counting.

The electrode wires of the counting tube are clipped to the high-voltage leads of the Nuclear-Chicago model 182 scaling unit. Direct current voltage is applied, and the scaling unit records both counts and time.

Each Bernstein-Ballentine tube has an optimum voltage for counting. This optimum voltage is the midpoint in the plateau in the curve of voltage versus counts per minute. In this plateau region, the number of counts registered in the scaling unit is proportional to the number of nuclear disintegrations in the carbon dioxide in the counting tube. Each tube is calibrated (48), and the counting voltage determined before using the tube for collecting experimental data. Figure 19 shows a typical counting plateau.

CALCULATION OF SPECIFIC ACTIVITY

A sample calculation follows, along with a note explaining each step.

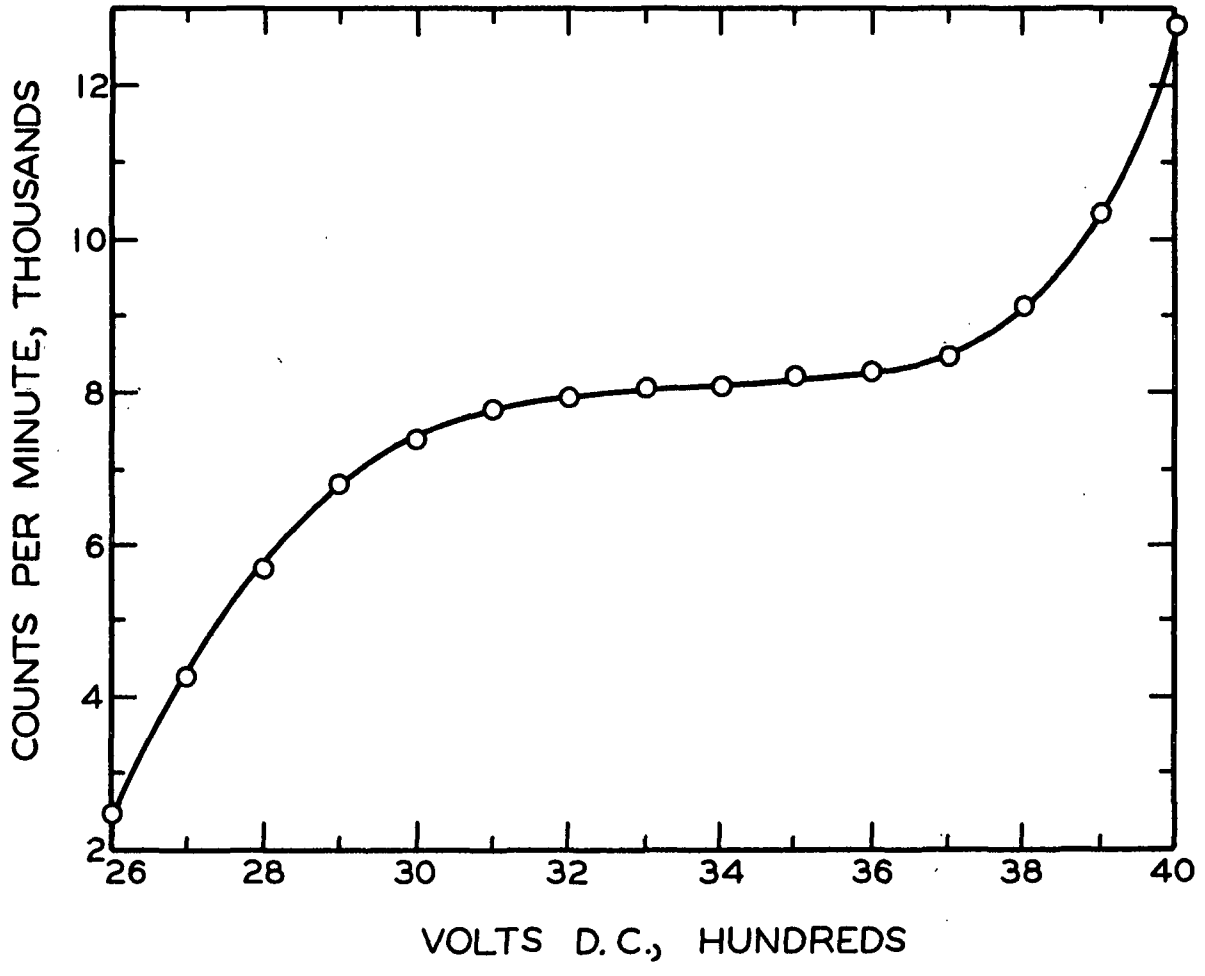

Figure 19. Counting Plateau, Bernstein-Ballentine Reference Tube

Sample: Isotope dilution of MBG after 18 hours' reaction in oxidation 8.

$\underline{P}_1$	= 316.9 mm. Hg	Pressure from manometer of Van Slyke apparatus with the volume of the carbon dioxide adjusted to 10 ml. in the gas buret.
$\underline{P}_2$	= 66.4 mm. Hg	Pressure from manometer of Van Slyke apparatus after carbon dioxide was transferred to tube. (corrects for water vapor)
$\underline{P}$	= 250.5 mm. Hg	$\underline{P}_1 - \underline{P}_2$ for the above run.
$\underline{c}$	= 5.4 mm. Hg	$\underline{P}_1 - \underline{P}_2$ from a blank run.
$\underline{P}_{CO_2}$	= 245.1 mm. Hg	Corrected pressure of carbon dioxide.
$\underline{T}$	= 24.6°C.	Temperature of water bath surrounding gas buret.
Factor	= 0.006810	Van Slyke's factor for 24.6°C. when the volume of the carbon dioxide is adjusted to 10 ml. (46).
C	= 1.67 mg.	$(\underline{P}_{CO_2})$(factor).
Known C	= 1.71 mg.	Calculated from the weight of the sample.
C found	= 97.7%	Experimental mg. C/calculated mg. C.
Volts	= 3300	Midpoint of counting plateau for tube used.
Time	= 42 min.	Time the tube was counted on the scaling unit.
Count	= 25,884	Counts registered on the scaling unit.
c.p.m.	= 616 ± 6	Counts per min. 90% confidence limits for 25,884 counts are ± 1.03% of the observed rate[1], or (0.0103)(616) = 6.4 c.p.m. Rounded off to 6 c.p.m.
Backgrd.	= 144 ± 2	Background count observed in a blank run.
Net c.p.m.	472 ± 6.3	$(\underline{A} \pm \underline{a}) - (\underline{B} \pm \underline{b}) = (\underline{A} - \underline{B}) \pm (\underline{a}^2 + \underline{b}^2)^{1/2}$ (50)
dis./(min.)	= 571 ± 7.6	Disintegrations per minute = counts per minute/$\underline{V}_r\underline{E}$. ($\underline{V}_r\underline{E}$ = 0.8263 for the tube used here)[2]
dis./(min.)(mg. C)	= 334 ± 4.4	Disintegrations per minute per calculated mg. C.
Specific activity	= 334 ± 4	Rounding off above value.

[1] For 90% confidence, the per cent error in the observed counting rate is (50):

$$\text{error } \% = \frac{165}{(\text{total count})^{1/2}} .$$

[2] $\underline{V}_r$ is a correction representing the fraction of the counting tube that is included in the space enclosed by the silvered portion of the walls (48). Each tube is calibrated for this. For this tube, $\underline{V}_r$ = 0.8432. $\underline{E}$ is an efficiency factor for the tube (48). $\underline{E}$ was assumed to be 0.98 for all the tubes used in this study.

COUNTING ACCURACY AND PRECISION

Having two measures of the amount of carbon in the sample provided a check on the method. Excluding the few runs in which the amount of carbon found using the Van Slyke apparatus was less than 90% or more than 110% of the amount calculated from the sample weight, the average per cent of carbon found in 123 combustions in the Van Slyke apparatus was 97.1% of the amount calculated from the weight. Two thirds of these results fell in the range 94 to 100%.

The amount of carbon calculated from the sample weight was used in the calculation of the specific activity. There were two reasons for using this amount. The samples, usually about 4 milligrams total weight, or about 2 mg. of carbon, were weighed on a semimicro balance. The weighings were accurate to $\pm$ 1% or better. Also, it was felt that the Van Slyke wet combustions were quantitative and produced the correct amount of carbon dioxide in the gas buret, but that the buret and manometer readings were somewhat inaccurate. Evidence for this is the fact that the scatter in specific activity data on duplicate samples was often less than the scatter in the Van Slyke manometric data. Table XVI shows the amount of carbon found and the activity for sets of combustions.

The accuracy was checked using a sample of one microcurie of sodium bicarbonate from Fischer Scientific Company. The accuracy of its radio assay was not known but it was reasonable to assume that it was within the range 1.0 $\pm$ 0.05 microcuries. The sample was converted to sodium carbonate and diluted to give a calculated activity of 555 $\pm$ 28 disintegrations per minute per milligram of carbon. Three samples, E, Table XVI, were counted. They gave an average of 523 dis./(min.)(mg. C). The experimental activity was thus 94.2% of the calculated activity.

TABLE XVI

VAN SLYKE RESULTS AND ACTIVITIES

Sample	Tube	Date	Carbon Weighed, mg.	Carbon Found, mg.	Percentage, found / weighed	Activity, dis. / (min.)(mg. C)
A			2.22	2.11	95.0	1769
			2.11	2.06	97.6	1761
B			2.27	2.21	97.4	1752
			2.20	2.16	98.2	1725
C			2.31	2.25	97.4	1576
			2.31	2.35	101.7	1574
D	1	8/17	0.95	0.89	93.7	110935
	1	8/17	0.95	0.92	96.8	112453
	6	8/17	0.95	0.92	96.8	113333
	6	8/17	0.95	0.92	96.8	113608
	6	11/26	1.675	1.60	95.5	110600
	6	11/26	1.675	1.59	94.9	113900
E			2.00	1.93	96.5	533
			2.00	1.97	98.5	529
			2.00	1.86	93.0	508

Samples A, B, and C: Methyl p-nitrobenzoate from isotope dilutions of methanol.
Sample D: Methyl β-D-glucoside(aglucone-^{14}C) starting material.
Sample E: Na_2CO_3(^{14}C).

The final activity of the MBG* was 71% of that calculated from the dilution of the glucose(^{14}C) from which it was made. The final activity of the M*BG was 106% of that calculated from the dilution of the methanol(^{14}C) from which it was made. It would be unreasonable to assume that the counting efficiency was 71% one time and 106% the next time. Evidently the commercial samples did not contain the correct amount of labeled material.

It may be concluded that the accuracy of the counting was near 100% and that the precision of the counting was about ± 2%.

The accuracy of the experimental results does not depend upon high accuracy in the counting, but upon high precision in the counting. In other words, the relative change in activity between starting material and isotopically diluted product is the important factor.[1]

The scaling unit can distinguish between two disintegrations only if they are separated by a given time, called the resolution time. Otherwise, only one count is registered. These losses in count are called coincidence losses. Using two samples of the same material, one very active and the other greatly diluted, the resolution time and coincidence losses were calculated. The resolution time was found to be 10 microseconds. This would lead to a coincidence loss of 1.7% at a counting rate of 100,000 counts per minute. The actual activities in the samples studied gave counting rates of less than 10,000 counts per minute. Coincidence losses were therefore negligible.

[1] If both the starting material specific activity, A, and the isotopically diluted sample specific activity, B, are inaccurate by the same factor, f, the isotope dilution calculations are not affected.

$$\underline{\text{Starting Material}} \qquad \underline{\text{Dilution}} \qquad \underline{\text{Diluted Sample}}$$

$$(\underline{X}\text{ mg.})(\underline{A})(\underline{f}) \quad + \quad (\underline{Y}\text{ mg.})(0) \quad = \quad (\underline{X} + \underline{Y})(\underline{B})(\underline{f})$$

$$X = \frac{YBf}{Af - Bf} = \frac{YB}{A - B} \tag{10}$$

The result is the same as in Equation (3), page 13.

APPENDIX III

CHROMATOGRAPHY

Three chromatographic developers were used in this study; they were:

A. n-Butanol – pyridine – water, (6:2:3), upper phase,

B. n-Butanol – acetic acid – water, (4:1:5), upper phase, and

C. Ethyl acetate – pyridine – water, (8:2:1).

Two spray reagents were used; they were:

A. p-Anisidine hydrochloride (51), made by mixing, in order, 0.5 g. p-anisidine hydrochloride, 5 ml. water, 10 ml. absolute ethanol, and 85 ml. n-butanol. The chromatograms were sprayed with this solution and then placed in an oven at 105°C. for five minutes. Hexoses gave a brown color (yellow for trace amounts), pentoses a pink color, and chlorates a purple color. MBG is not usually detected, but if very heavy may be seen under ultraviolet light as a spot which is lighter than the background. A trace of sodium hydrosulfite added with the p-anisidine will prevent the solution from turning dark red.

B. Silver nitrate, after (52), which consists of three solutions:

1. Silver nitrate, made by dissolving 6 g. of silver nitrate in 11 ml. of water, and then adding 190 ml. of acetone.
2. Sodium hydroxide, made by dissolving 2 g. of sodium hydroxide in 5 ml. of water, and then adding 95 ml. of absolute ethanol.
3. Ten per cent aqueous sodium thiosulfate.

These solutions could be sprayed on the chromatograms or used as a dip. Best results were obtained using them as a dip for Whatman number one chromatographic paper, and as a spray for the very heavy Whatman number 17 paper.

When used as a dip, the chromatogram was dipped slowly through the silver nitrate, dried, dipped through the sodium hydroxide, allowed to stand 10 minutes, dipped through the thiosulfate, washed with water, and dried.

When used as a spray, better results were obtained if the chromatogram was sprayed several times with the sodium hydroxide solution.

All organic materials in this study gave a brown-black spot in a lighter-brown background. Occasionally, a salt spot was lighter in color than the background.

This silver nitrate spray reagent was very sensitive, as shown in Table XVII.

TABLE XVII

CHROMATOGRAPHIC SENSITIVITY

Material	Amount, micrograms	Spot Size
MBG	1.0	very faint
	0.5	not found
Glucose	1.0	medium
	0.25	faint

It was found that as much as 1000 micrograms of MBG could be placed on a single spot on Whatman number one paper without causing the chromatogram to be streaked. Putting this much MBG in one spot, and being able to detect such small amounts of glucose, gave a very efficient test for glucose impurity in the MBG.

A wick of Whatman number one paper was sewed across the top of the very heavy Whatman number 17 paper to slow the flow of solvent onto the sheet. As much as 80 mg. of solids could be streaked on each inch of starting line on the

heavy paper. R_f values were not used to identify spots on the chromatograms. When there was any chance that identification would be difficult, a known compound was spotted on the same chromatogram for reference.

APPENDIX IV

AQUEOUS CHLORINE

AQUEOUS CHLORINE EQUILIBRIA

Chlorine in aqueous solution undergoes hydrolysis to hypochlorous acid, which, in turn, may dissociate into hypochlorite ions and hydrogen ions. These reactions are:

$$Cl_{2(aq)} + H_2O \longrightarrow HOCl + H^+ + Cl^- \qquad (11).$$

$$HOCl \longrightarrow H^+ + OCl^- \qquad (12).$$

The equilibrium constants for the two reactions are known. The constant for Reaction (11) is $3.9(10^{-4})$ at 25°C. (53), and for Reaction (12) is $3.2(10^{-8})$ at 25°C. (54). Reactions analogous to (11) and (12) occur when chlorine is passed into sodium hydroxide solutions to form sodium hypochlorite. Figure 20, based on Spalding's calculations of the ionic and molecular concentrations for a solution 0.04M in total chlorine at 25°C., illustrates the chlorine-water equilibrium (55).

Other reactions may occur in aqueous chlorine solutions. The reactions of main concern in this study were the ones leading to the disproportionation of available chlorine into chloride and chlorate ions. The disproportionation is most rapid at pH 7 (16, 56). There have been many investigations of this chlorate formation and several proposed mechanisms (57-63). The over-all result of each set of these reactions may be expressed as:

$$3\ OCl^- \longrightarrow 2\ Cl^- + ClO_3^- \qquad (13).$$

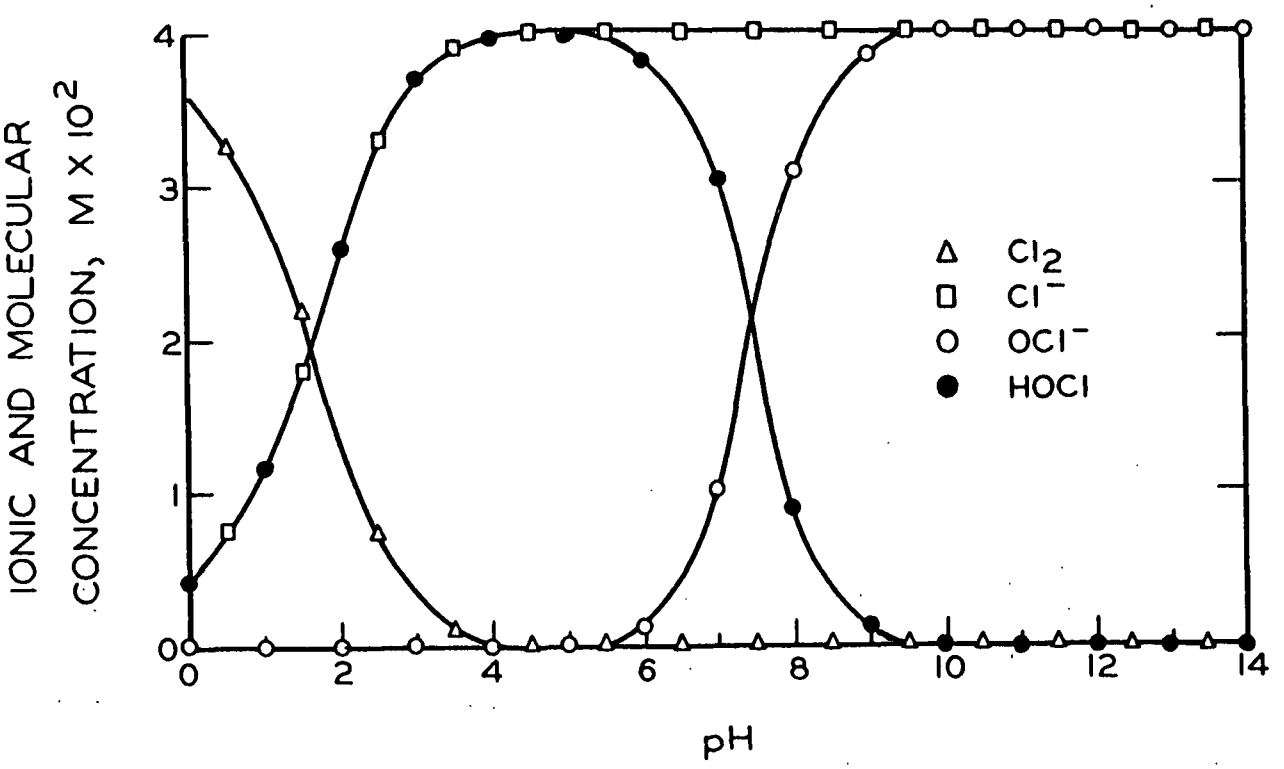

Figure 20. Distribution of Molecular and Ionic Species in 0.04M Aqueous Chlorine at 25°C. (55)

Lister (60-63), in studying sodium hypochlorite solutions, has studied this disproportionation as thoroughly as anyone. He concluded that the disproportionation proceeds through the reactions:

$$2\ HOCl \rightleftarrows HClO_2 + Cl^- + H^+ \qquad (14)$$

$$HClO_2 \rightleftarrows ClO_2^- + H^+ \qquad (15)$$

$$HOCl + ClO_2^- \rightleftarrows HClO_3 + Cl^- \qquad (16)$$

$$HClO_3 \rightleftarrows ClO_3^- + H^+ \qquad (17)$$

These reactions, together with the reverse of (12) may be added to give the overall result (13). Lister also found oxygen could be formed to a minor extent (63):

$$HOCl \longrightarrow H^+ + Cl^- + 1/2\ O_2 \qquad (18).$$

The mechanism of chlorate formation was not investigated in this study. The loss of available chlorine during the equilibrium, Fig. 4, was evidence of its formation. Other evidence will be discussed later.

Grillo (9) does not mention chlorate specifically, but he did find that it was necessary to correct for loss of available chlorine when working at pH 4.6 and 6.5. At pH 2.1, he found that no such correction was necessary. His correction at pH 4.6 involved application of a rate constant for the loss of oxidant in blank solutions. At pH 6.5, he found this rate constant to be approximately third order, and he applied a point-by-point correction. His larger correction at pH 6.5 is understandable because chlorate formation is most rapid at pH 7.

Whistler and Schweiger (16) found 29% of their hypochlorite was converted to chlorate at pH 7, with much less conversion at other pH values.

Theander (7) corrected his oxidant consumption for the loss of active oxidant due to the formation of chlorate which he found most rapid at pH 7. He did not determine chlorate during the actual oxidation runs because the reaction products interfered with the determinations, but he did determine it on blank runs with no glucoside present. He found about 5% of the active oxidant was converted to chlorate per hour, at pH 7 and 30°C.

In this study, the rate of loss of available chlorine due to chlorate formation during the oxidation of the MBG, Fig. 15, did not follow second or third-order kinetics closely. In fact, an approximation of the curve is given by the empirical equation:

$$-\frac{d(Ox)}{dt} = \frac{(Ox)^4}{3} \qquad (19),$$

where the units are liters, moles, and seconds. This empirical equation does not apply to the equilibration period.

ANALYSIS OF THE CHLORINE SOLUTIONS

The methods of White (64) for the determination of total available chlorine, total oxidizing power (to determine the amount of chlorate), and chloride were used in this study. The 0.1N sodium thiosulfate solution, made according to Spalding's method (55), was extremely stable. Its concentration changed less than 1% in a year.

OXIDANT PREPARATION AND EQUILIBRATION

For the three main oxidations, a fresh stock solution was made by passing chlorine gas through 0.47N sodium hydroxide at 2-6°C. until the pH of the solution dropped to about 6. Aliquots of this stock solution were titrated to determine the available chlorine content. These stock solutions were about 0.8N in available chlorine.

A portion of the stock solution was added to the reaction flask and diluted alternately with water and 0.1N sodium hydroxide until the pH reached 7 at the same time the desired volume was reached. During this dilution, the temperature was reaching 25°C. The dilution procedure required 30 min. to one hour. Enough stock solution was used to give a diluted solution about 0.45N in available chlorine at 25°C. and pH 7. The sodium hydroxide used in Oxidations 7 and 8 was carbonate free. It was made by allowing the sodium carbonate to settle out of a 50% sodium hydroxide solution for one month. Starting time for the equilibration was taken as the time the dilution was completed.

EVIDENCE OF CHLORATE FORMATION

In the preliminary work, two methods were used for finding the total amount of chlorine in the solutions. The first method determined available chlorine by an iodometric method, titrating with thiosulfate the iodine which is released by the available chlorine. Assuming no chlorate formation or other complications, the available chlorine content is numerically the same as the total chlorine content. The second method used silver nitrate to titrate after first using hydrogen peroxide to convert available chlorine to chloride. Again, assuming no chlorate formation or other side reaction, this method would find all of the chlorine present. Therefore, in solutions containing only $Cl_{2(aq)}$, HOCl, OCl^-, and Cl^-, both titrations determine the total chlorine present. The first method was used during the main oxidations.

Chlorine was absorbed in distilled water at 4°C. and in a sodium hydroxide solution at 4°C. Initially, for both solutions, there was agreement between the two titration methods. However, upon standing at room temperature, these solutions began to give lower and lower results by both methods. The effect was more pronounced for the sodium hydroxide solution and for the available chlorine titration. Modification of the available chlorine titration so it would also detect chlorate showed that most of the apparent loss was due to chlorate formation.

In another test, chlorine was absorbed in a sodium hydroxide solution at 10°C. in an opaque flask. The initial titration showed some chlorate had been formed at 10°C.; the temperature has to be below 9.6°C. to prevent chlorate formation (65). The chlorine water was placed in a 25°C. bath and the titrations and pH followed for 22 hours (Fig. 21). A chlorate determination at the end of this time showed the apparent loss of chlorine was due to chlorate formation.

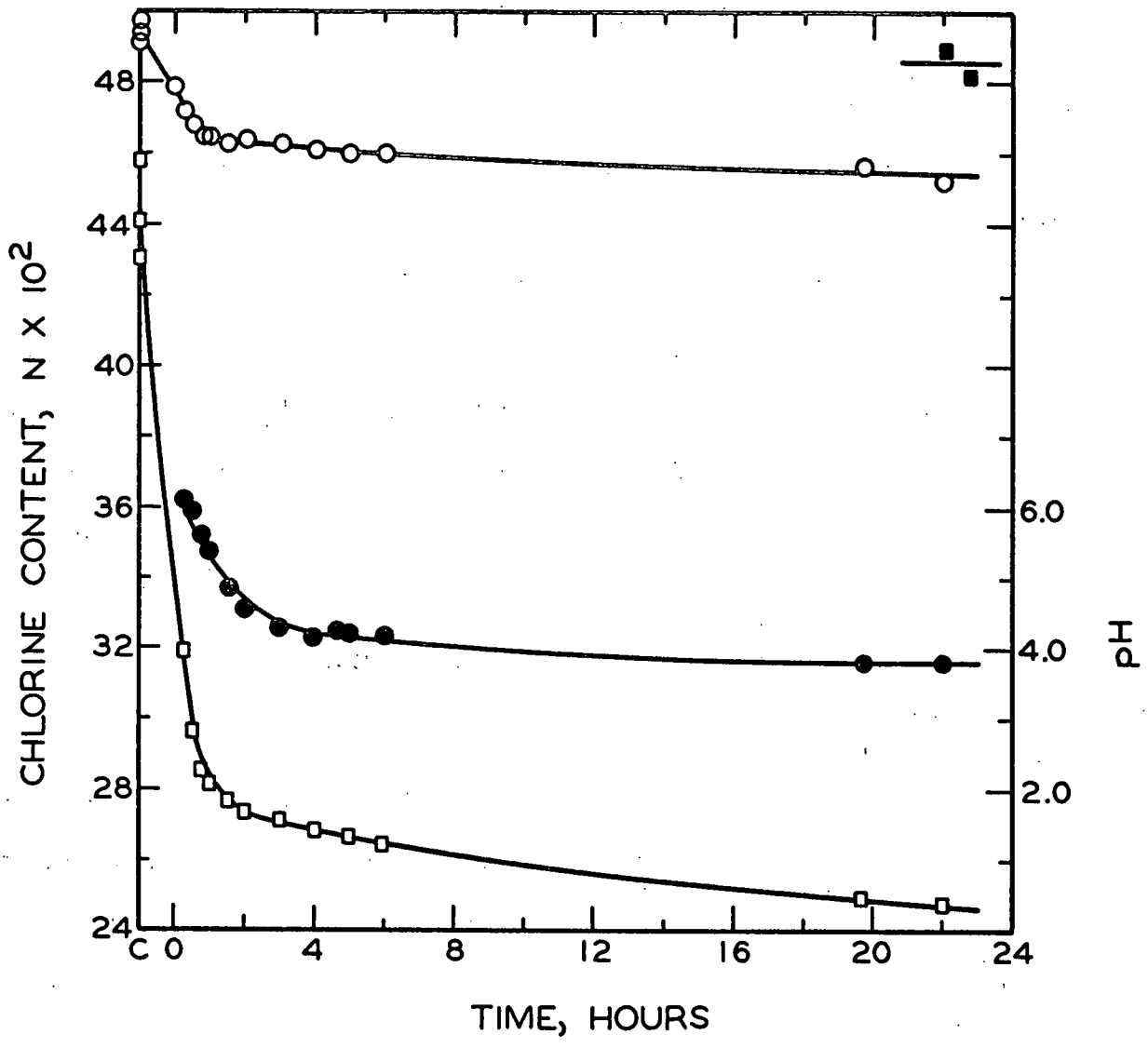

Figure 21. Chlorine Concentrations by Different Titrations, and pH
● pH, C Solution at 10°C. Before Warming to 25°C.
Titrations: o Silver Nitrate, □ Available Chlorine, ■ Chlorate

It can be shown that the apparent loss in chlorine due to chlorate formation is one sixth as large when titrating with silver nitrate as when titrating with thiosulfate for available chlorine. Figure 21 shows this was approximately the case in the above test.

The above discussion has considered only chlorate formation. It is also possible to have chlorites, chlorous acid, and chlorine dioxide present in the system, further complicating it.

In the above experiment there was no sodium hydroxide added to control the pH. Because chlorate formation is most rapid at pH 7, it would be expected that chlorate formation in the oxidations at pH 7 was even faster.

There was also chromatographic evidence of the formation of chlorate. Calculations showed the oxidation solutions, after completion of the oxidation, contained approximately 12 g. per liter of sodium chlorate, plus other salts. Often some salts remained in the samples chromatographed. It was found that chlorate gave a purple color with p-anisidine hydrochloride spray. In chromatographic developer A, Appendix III, chlorate formed two adjacent spots, a faint faster one followed by a slower, heavier one. In developer B, the fainter spot overlapped the heavy spot and moved only slightly faster. In developer C, there was only one spot, but its leading edge was considerably heavier than the rest of the spot.

The behavior of the oxidation solutions also indicated the presence of chlorate. In the first attempts to separate formic acid by distillation, the solution was made acidic and distilled at about 150°C. The distillate was yellow, indicating some form of available chlorine had been produced. Possible reasons were a reversal of Reactions (14) through (17), or the formation of chlorine dioxide from chlorate and the acid (66). Similarly, in the first attempts to hydrolyze samples of the oxidation solutions with 2N hydrochloric acid at 80°C., a yellow color developed. This was later prevented by using some sulfurous acid in the hydrolysis.

APPENDIX V

PRELIMINARY OXIDATIONS

OXIDATION OF UNLABELED METHYL β-D-GLUCOPYRANOSIDE

OXIDATION ONE

Oxidation one was a 14-hour blank run, using only the hypochlorite solution in the reaction flask. Its purpose was to test the equipment and to see if the hypochlorite solution caused any neutral compounds to be formed from the rubber in the combination electrode. After the oxidation, the solution was deionized with IR-120 and IR-45 ion-exchange resins, and examined chromatographically. No neutral compounds were formed. Therefore, chromatograms in the later oxidations would not be complicated from this source. It was decided that 0.10N sodium hydroxide was too dilute for use in the pH control apparatus.

OXIDATION TWO

A freshly prepared aqueous chlorine solution was used in the oxidation of 1.0 g. of MBG. There was no equilibration of the oxidant prior to the oxidation. The concentration of available chlorine was calculated to be 0.103N, but this neglected chlorate formation. After 12 hours, the oxidation was stopped with sulfurous acid. It was found that the salts present in the solution interfered with proper development of chromatograms, even after an attempt at removing the salts by precipitation with barium and silver carbonate. After ion exchange with IR-120 and IR-45 resins, only unreacted MBG was found chromatographically. The sodium hydroxide for pH control was 0.5N; this was still somewhat too dilute.

OXIDATION THREE

This oxidation was similar to the previous one, except that the concentration of available chlorine in the oxidant was calculated to be 0.334N, again neglecting chlorate formation. Over 80% of the available chlorine was consumed during the oxidation. The sodium hydroxide for pH control was 1.0N.

The oxidation products were fractionated according to Henderson's method (Appendix VIII) into a neutral fraction and an acidic fraction. The fractions were investigated chromatographically using developers A and B of Appendix III, and alkaline silver nitrate and p-anisidine hydrochloride sprays. Chromatograms of the neutral fraction showed spots for 8 to 13 products, depending upon the manner of development. Chromatograms of the acidic fraction showed 12 to 13 spots, but some of these were later found to be salts. Identification of these chromatographic spots was not made. Chromatographing without prior salt removal failed.

This oxidation showed that there is a considerable oxidation in 18 hours with many products, that the fractionation procedure did separate the neutral and the acidic compounds, that chromatography without salt removal was not possible, and that 1.0N sodium hydroxide solution was satisfactory for pH control. The large consumption of available chlorine led to investigations, some of which are discussed in Appendix IV, which showed that much more chlorate was being formed.

OXIDATION FOUR

This oxidation was made to see if chlorate ion oxidized MBG. One gram of MBG was dissolved in 250 ml. of 0.07M potassium chlorate at 25°C., and the pH

adjusted. One sample of the solution was withdrawn after 18.5 hours and stored by freezing; the remainder of the solution reacted for a total of 48 hours before it was removed from the flask and frozen. The pH was 6.7 for the first sample and 6.8 for the second.

Both samples were fractionated by Henderson's method. Enough of the 48-hour sample was fractionated to contain 200 mg. of the starting MBG. The dry solids from the neutral fraction weighed 186 mg. and was shown chromatographically to contain only MBG. Chromatograms of the 26 mg. of sirup obtained in the acidic fraction were heavily streaked with salts. Salts came through the fractionation procedure from the 18.5-hour sample and made chromatographic investigation difficult. Glucose and one additional compound were found in the neutral fraction, but there was a trace of glucose and one other impurity in the starting MBG.

It was concluded that chlorate is not an oxidant for MBG in the hypochlorite solutions. This agrees with the conclusion of Whistler and Schweiger for the hypochlorite oxidation of amylopectin (16).

The fact that glucose did appear in the neutral fraction of the 18.5-hour sample but not in the 48-hour sample raised some doubts about the efficiency of the fractionation procedure. It was concluded that it would not be safe to fractionate before isotope dilution because the results depend upon 100% efficiency in the fractionation.

OXIDATION FIVE, METHYL β-D-GLUCOPYRANOSIDE(GLUCOSYL-^{14}C)

STARTING MATERIAL

The recrystallized MBG* described in Table III was the starting material for this oxidation.

OXIDATION

The aqueous chlorine solution was allowed to equilibrate 21.25 hours at 25°C. and pH 7 before adding the MBG*. The change in available chlorine concentration during this equilibration is shown in Fig. 4. After this equilibration, 0.6806 g. of MBG* was added in water solution, diluting the starting concentration of available chlorine to 0.065N. After 18 hours' oxidation, when the available chlorine concentration was 0.039N, the reaction was stopped with sulfurous acid and the pH readjusted with sodium hydroxide.

ANALYSIS

Carbon Dioxide

The conditions for efficient carbon dioxide removal had to be worked out using the oxidation solution from this run, since it was the first one using labeled starting material. A satisfactory analysis was not completed, but it was learned that an aliquot of the liquid and vapor should be used instead of trying to remove the carbon dioxide from the original reaction flask. Temperature and pH requirements for proper carbon dioxide removal were also worked out. This work led to a test of the method using $Na_2CO_3(^{14}C)$, as described in Appendix VI.

Fractionation

Part of the solution was fractionated according to Henderson's method. Unreacted MBG*, glucose, arabinose, and four unidentified compounds were found chromatographically in the neutral fraction. Again, there was difficulty with salts in the acidic fraction, especially ammonium sulfate. This was the last attempt to use the fractionation procedure.

Isotope Dilution

To 10-ml. samples of the oxidation solution, unlabeled glucose, arabinose, and MBG were added for isotope dilution. The crystals formed in the solutions were separated from the mother liquors. Chromatographic investigation showed that most of the arabinose and all of the MBG remained in the mother liquor, the crystals being salts. The glucose was distributed in both the crystals and the mother liquor. These results led to investigations of salt removal by extraction and chromatography, as described in Appendix VI.

OXIDATION SEVEN, METHYL β-D-GLUCOPYRANOSIDE(AGLUCONE-^{14}C)

STARTING MATERIAL

The M*BG used in this oxidation was also used in oxidation eight, one of the two main oxidations in this study. The preparation and description of the M*BG is given in that part of the Experimental section dealing with Oxidation 8, page 36.

OXIDATION

The oxidant was prepared to be identical to that used in oxidation six, but the loss of available chlorine during the equilibration was slightly faster than for that oxidation, as seen in Fig. 4 and recorded in the data of Appendix IX. Consequently, after removing a sample for a blank and injecting 0.9206 g. of M*BG, the starting conditions were close to, but not identical with, those of oxidation six, Table XIII.

Available chlorine concentration was followed in both the oxidation solution and the blank solution during the oxidation. The data are shown in Appendix IX, and the results are shown in Fig. 6. Nine- and eighteen-hour samples were

taken; for the 9-hr. sample, 20% of both the liquid and gaseous phases was removed with a syringe and injected into an evacuated flask. In this oxidation, unlabeled methanol for isotope dilution was injected in the samples before storing in a refrigerator. Table XVIII shows the calculated solids content of the solutions, and Appendix IX describes the calculations.

TABLE XVIII

CALCULATED SOLIDS CONTENT OF OXIDATION SOLUTIONS

Solid	9-Hr. Solution[a]		18-Hr. Solution[b]	
	grams	mg./ml.	grams	mg./ml.
NaCl	1.55	14.98	5.62	14.38
$NaClO_3$	1.20	11.59	4.68	11.97
Na_2SO_4	0.25	2.42	0.77	1.97
Carbohydrates[c]	0.1755	1.695	0.666	1.704
Methanol	0.155	1.498	0.593	1.517

[a]Total volume: 103.5 ml. liquid phase and 146.5 ml. gaseous phase.
[b]Total volume: 390.9 ml. liquid phase and 695.1 ml. gaseous phase.
[c]Calculated as unreacted M*BG.

ANALYSIS

Carbon Dioxide

For carbon dioxide determinations, 22.67% of the 9-hr. oxidation solution and 26.62% of the vapor, and 10% of the 18-hr. oxidation solution and vapor were analyzed according to the method of Appendix VI. The results showed that 0.011 $\pm$ 0.008% of the aglucone carbon appeared as carbon dioxide after 9 hours of oxidation, and 0.037 $\pm$ 0.006% after 18 hours.

The low yield of labeled carbon dioxide caused the samples counted to be only weakly active. Therefore, the confidence limits on the counts were relatively large. Data are shown in Appendix IX.

Methanol

The analyses for methanol in the oxidation solutions were made using the method described in Appendix VI. In that appendix, in the discussion of the calculations for the method, the results for this oxidation are given. The calculations showed that 4.15 ± 0.04% of the aglucone carbon appeared as methanol after 9 hours' oxidation, and 6.28 ± 0.05% after 18 hours.

Unreacted Glucoside

The method for these analyses is given in Appendix VI, and the results in Table XXV in that appendix. The averages for duplicate results showed 84.2% of the starting M*BG remained after 9 hours' oxidation, and 82.5% after 18 hours. The amount of M*BG oxidized was, therefore, 15.8% at 9 hours and 17.5% at 18 hours.

APPENDIX VI

METHODS OF ANALYSIS

METHOD OF DETERMINATION OF CARBON DIOXIDE

Carbon dioxide oxidation product was determined by making use of its radioactivity. This method is therefore limited to finding the carbon dioxide which originated in labeled starting materials. In this study, the labeled starting material was the glucosyl portion of MBG in one case, and the aglucone portion in another case.

Figure 22 shows the apparatus used for removing the carbon dioxide from a sample and for absorbing it in sodium hydroxide solution. Other apparatus used was the Van Slyke manometric apparatus and Bernstein-Ballentine counting tubes described in Appendix II.

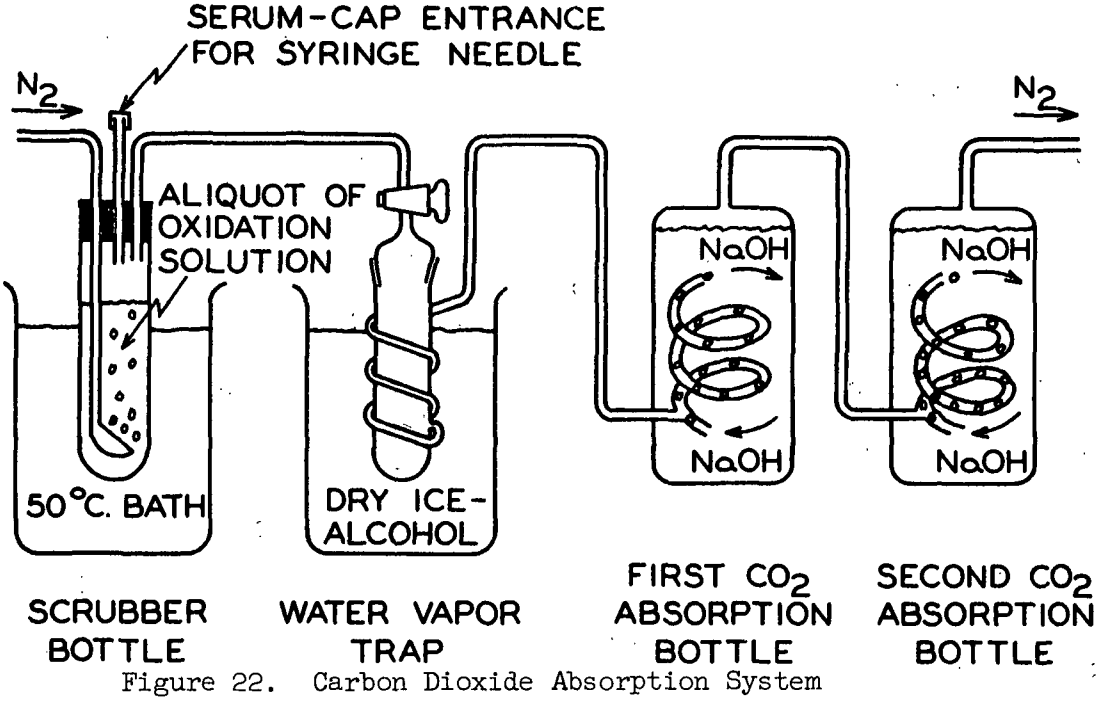

Figure 22. Carbon Dioxide Absorption System

An aliquot, appropriate amounts of both the liquid and gaseous phases, from the oxidation flask or storage flask[1], was transferred by syringe into the scrubber bottle of the carbon dioxide absorption system. For each 25 ml. of liquid, 5 drops each of bromothymol blue and Universal wide range indicator were injected. These indicators made it easy to control the pH of the solution in the range 5.5 to 6.5. The pH was held in this range by adding dilute sulfuric acid by syringe. The pH of the solution increases as carbon dioxide is removed. Without the addition of dilute acid to keep the solution slightly acidic, the carbon dioxide will not be quantitatively removed. The color of the solution with the indicators was yellow-orange at pH 5.5 and yellow-green at pH 6.5.

The scrubber bottle was immersed in a 50°C. constant-temperature bath[2]. Prepurified nitrogen was slowly passed through the system at a rate causing the spiral in the two Vanier absorption bottles to contain alternate moving bands of gas and caustic solution. At no time during this study did any carbon dioxide reach the second bottle; the first absorption bottle was 100% efficient in absorbing carbon dioxide(^{14}C). A water vapor trap was placed in the system before the first absorption bottle to prevent dilution of the caustic solution by water vapor from the warm sample.

The sodium hydroxide solution in the absorption bottle was 0.815N, the same concentration as is required for use in the Van Slyke manometric apparatus.

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[1] In Oxidation 8, aliquot samples of both the liquid and gaseous phases from the reaction flask were removed at various times with a syringe and injected into evacuated storage flasks. Determination of carbon dioxide could therefore be made on all the samples from Oxidation 8.

[2] The bath was at 60°C. in the analysis for Oxidation 6 and for the test of the method using Na_2CO_3(^{14}C). Also, no indicators were used in the latter case.

Exactly 50 ml. of the sodium hydroxide solution was placed in each bottle at the start of each determination. Periodically, the nitrogen flow was stopped and two milliliter aliquots were withdrawn from the absorption bottles using a syringe which had a small Tygon tube pushed over the needle. This sample was injected in the gas buret of the Van Slyke apparatus. To do this, the mercury level in the clean Van Slyke apparatus was lowered so about 5 ml. of air was in the gas buret above the mercury. The plug of the stopcock at the top of the gas buret was removed. With the help of a wire guide, the small Tygon tube on the needle of the syringe was inserted in the gas buret and the 2-ml. sample was injected (Fig. 23).

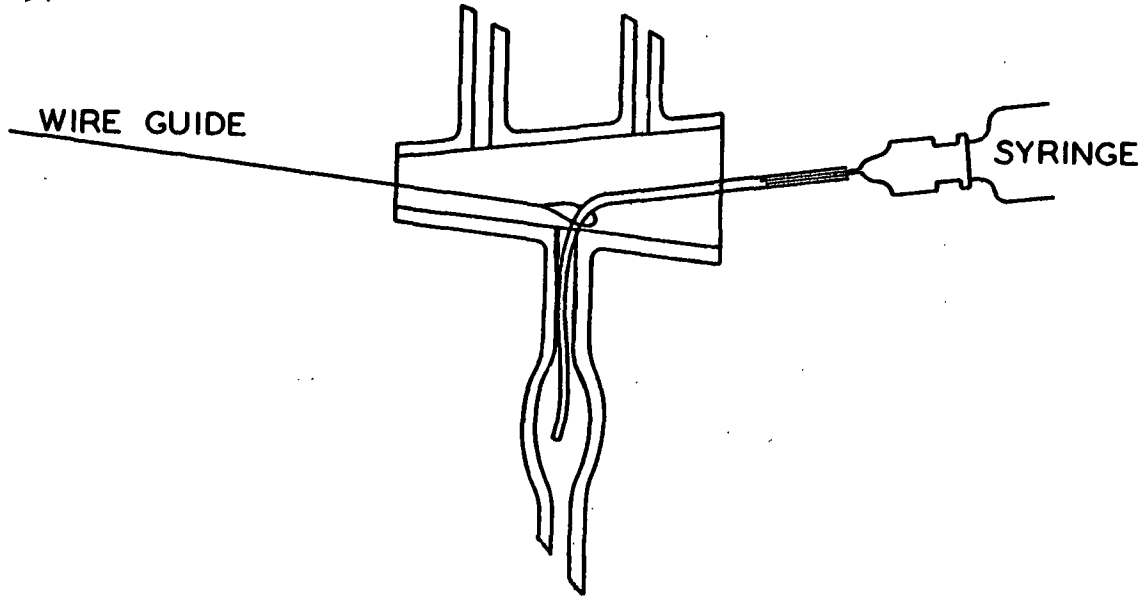

Figure 23. Insertion of Sample in Van Slyke Apparatus

The stopcock plug was regreased and replaced. The air above the sample was expelled as is always done in the Van Slyke procedure. From this point on, the procedure followed the normal Van Slyke procedure (46), briefly described in Appendix II, beginning with the liberation of the carbon dioxide by acidifying the sample solution with lactic acid.

The manometric data were of doubtful value because the amount of carbon dioxide in these samples was so small. However, these data were unimportant. The liberated carbon dioxide was transferred into a Bernstein-Ballentine tube in the normal manner (Appendix II), and the activity determined. These activity data were good, and the very small amounts of carbon dioxide could be determined in this way.

An example of the data and calculations will best explain the type of results obtained. The data collected and the calculations made during the analysis for carbon dioxide in the 18-hr. oxidation solution of Oxidation 6 are shown in Table XIX.

The activity of the starting material was 15,800 dis./(min.)(mg. glucosyl carbon) (Table III). Therefore, 7102 $\pm$ 132 dis./min. represents 0.4495 $\pm$ 0.0084 mg. of glucosyl carbon in the sample as carbon dioxide. The sample taken was 10% of the contents of the oxidation flask. Therefore, the sample contained (0.10)(608.2) mg. of carbohydrates (Table IV), or 22.56 mg. of glucosyl carbon. The percentage of glucosyl carbon appearing as carbon dioxide during the reaction was therefore (0.4495 $\pm$ 0.0084)(100)/22.56 or 1.99 $\pm$ 0.04%.

In a test of this method of carbon dioxide determination, a solution of sodium carbonate(^{14}C) of known activity was analyzed. It was known that the activity which should have been found in the absorption bottles was 1119 disintegrations per min. Using calculations similar to those given in Table XIX, the results shown in Table XX were obtained. The method was thus shown to be accurate to within at least $\pm$ 2%.

TABLE XIX

CARBON DIOXIDE DETERMINATION, OXIDATION SIX

Nitrogen Flow, hr.	Net Counts/Min.[a] from 2 Ml. of Abs. Bottle 1	Dis./Min.[b] from 2 Ml. of Abs. Bottle 1	Calculation[c]	Dis./Min. in Sample
12	181 ± 5	214 ± 6	x(50/2) + 0	5350 ± 150
19	244 ± 5	289 ± 6	x(48/2) + 214	7150 ± 144
19	240 ± 7	284 ± 8	x(46/2) + 503	7035 ± 161
24	237 ± 4	280 ± 5	x(44/2) + 787	6947 ± 110
40	246 ± 5	291 ± 6	x(42/2) + 1067	7178 ± 126
40	247 ± 5	292 ± 6	x(40/2) + 1358	7198 ± 120
40	-4 ± 4[d]	0[d]	--	--
			Average (excluding 12-hr. count):	7102 ± 132

[a]Net counts/min. = gross counts/min. - background counts/min. (see page 80). The "±" terms shows 90% confidence limits on the count.

[b]Net count corrected by the quantity $\underline{V_r E}$, footnote 2, page 80.

[c]The first aliquot counted was 2 ml. out of the original 50 ml. The second aliquot was 2 ml. out of the remaining 48 ml.; then to this was added the activity removed in the first aliquot, etc.

[d]This entry is for absorption bottle 2.

TABLE XX

TEST OF CARBON DIOXIDE DETERMINATION METHOD

Nitrogen Flow, hr.	Dis./Min. Abs. Bottle 1	Dis./Min. Abs. Bottle 2	Percentage of Known
5.5	901	0	80.6
11.2	1097	-	98.0
19.0	1102	0	98.5
41.5	1185	-	105.9
41.5	1116	0	99.7

METHOD OF ISOTOPE DILUTION ANALYSIS FOR METHANOL

This method is a modification of those given by Cheronis and Entrikin (67), and by Wild (68), which convert the methanol to the methyl p-nitrobenzoate ester.

Isotope dilution was done by adding one milliliter of an aqueous methanol solution, containing 40 λ (31.7 mg.) of methanol per milliliter, to a 20-ml. aliquot of the oxidation solution in a 25-ml. flask[1]. This diluted sample was poured into a small distilling flask containing 32 g. anhydrous potassium carbonate. The flask was heated in a paraffin oil bath until approximately 0.5 ml. of distillate had been collected in a 4-ml. test tube. To this distillate was added 0.33 ml. of aqueous potassium hydroxide (0.5 g./ml.) and 0.17 ml. aqueous sodium acetate (0.25 g./ml.). The solution was cooled to near freezing in a dry ice-alcohol bath. To this cold solution was added one milliliter of p-nitrobenzoyl chloride solution (1 g. p-nitrobenzoyl chloride dissolved in 2 ml. of purified hexane and 8 ml. of benzene). The test tube was stoppered and the mixture agitated for 20 minutes. After cooling again, another 0.5 ml. of the p-nitrobenzoyl chloride solution was added. The mixture was agitated for another 20 minutes.

The mixture was transferred to a 60-ml. separatory funnel. The test tube was washed with 20 ml. of diethyl ether. The ether layer was extracted successively with 10 ml. distilled water, 10 ml. aqueous 5% sodium hydroxide, 10 ml. aqueous 5% hydrochloric acid, and 10 ml. distilled water[2]. The ether layer was transferred to a beaker where the solvents were allowed to evaporate. The product

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[1] In the earlier analyses, this dilution was made by adding 42 λ (33 mg.) of pure unlabeled methanol with a micropipet.

[2] If the aqueous layers are collected and combined, a precipitate of the p-nitrobenzoic acid by-product will form, melting point 242.4°C. (25).

was dissolved in warm ethanol. Darco G-60 activated carbon[1] was added and then filtered out using a medium sintered glass microfilter. A few drops of water were added. Upon cooling, crystals of methyl p-nitrobenzoate(methyl-^{14}C) formed. The crystals were filtered from the mother liquor and dried over calcium chloride in a desiccator. Their specific activity was then determined by the methods given in Appendix II, and the percentage of methanol calculated.

The method was applied in duplicate to the 18-hour oxidation solution from the oxidation of MBG*, Oxidation 6. After determining the activity of the crude ester samples, the samples were recrystallized and the activity again determined. The results were statistically the same. Table XXI shows the results.

TABLE XXI

ACTIVITY OF ESTER FROM METHANOL PRODUCED IN 18-HOUR OXIDATION OF MBG*

Methyl p-Nitrobenzoate Sample	Specific Activity, dis./(min.)(mg. C)
Crude 1	2.55 ± 1.42
Crude 2	2.84 ± 1.60
Average:	2.70 ± 1.51
Recrystallized 1	2.27 ± 1.59
Recrystallized 2	3.18 ± 1.70
Average:	2.73 ± 1.65
Over-all average:	2.71 ± 1.58

[1]Darco G-60 activated carbon is a product of Atlas Powder Company, Wilmington, Delaware.

The activity was so low that the count was barely above the background count. The statistical error (90% confidence limits) is therefore quite large.

The method was also applied to both oxidation solutions from the oxidation of M*BG, Oxidation 7. Duplicate ester samples from the 9-hr. oxidation gave 775 ± 10 and 737 ± 5 dis./(min.)(mg. C). From the 18-hr. sample, duplicate esters gave 1094 ± 11 and 1124 ± 8 dis./(min.)(mg. C).

Table XXII describes the calculations for determining the amounts of methanol when duplicate oxidations are made, one using MBG* and the other using M*BG. Using these calculations for the 18-hr. solutions of Oxidations 6 and 7 with:

$$\alpha_G = 15{,}800 \text{ dis./(min.)(mg. glucosyl C)},$$
$$\alpha_A = 788{,}200 \text{ dis./(min.)(mg. aglucone C)},$$
$$M = 227.2 \text{ mg. (an average for the two runs)}^1,$$
$$A_1 = 21.68 \pm 12.64 \text{ dis./(min.)(mg. C)}, \text{ and}$$
$$A_2 = 8872 \pm 76 \text{ dis./(min.)(mg. C)},$$

one finds that 2.58 ± 0.02 mg. of carbon from the aglucone group and 0.315 ± 0.182 mg. of carbon from the glucosyl group appeared as methanol oxidation product. This amounts to 6.28 ± 0.05% of the aglucone carbon and 0.14 ± 0.08% of the glucosyl carbon[2].

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[1] "M" in this case was calculated on the basis of the entire oxidation solution, not just the 20-ml. sample.

[2] Oxidations 6 and 7 were not exact duplicates (Fig. 15), but this will make no difference in the case of the calculation for the methanol originating in the glucosyl portion of the MBG, because that amount is so near zero that experimental errors are larger than those caused by slight differences in the oxidations. In the case of the methanol originating in the aglucone portion of the MBG, the above type of calculation is unnecessary. Neglecting the small amount of dilution from methanol originating in the glucosyl group, the methanol having its origin in the aglucone group was calculated to be 6.15 ± 0.05%. This result is not significantly different from the one obtained in the above calculations. This simplified calculation for the 9-hr. oxidation in Oxidation 7 showed 4.15 ± 0.04% of the aglucone carbon had become methanolic carbon.

TABLE XXII

CALCULATIONS FOR METHANOL ISOTOPE DILUTION (Unknown values are enclosed in parentheses)

Step (explained in text)	Run 1[a]	Run 2[a]
Starting material	Methyl β-D-glucoside (glucosyl-^{14}C)	Methyl β-D-glucoside (aglucone-^{14}C)
Activity of starting material	α_G, dis./(min.)(mg. glucosyl C)	α_A, dis./(min.)(mg. aglucone C)
Oxidize, take aliquot of oxidation solution containing:	MeOH from glucosyl: Mg. C (M_G); Activity α_G. MeOH from aglucone: Mg. C (M_A); Activity 0	MeOH from glucosyl: Mg. C (M_G); Activity 0. MeOH from aglucone: Mg. C (M_A); Activity α_A
Add unlabeled methanol (M mg. C) giving total MeOH (isotope dilution step).	Total MeOH: Mg. C $M + (M_G) + (M_A)$; Activity $\frac{(M_G)\,\alpha_G}{M + (M_G) + (M_A)}$	Total MeOH: Mg. C $M + (M_G) + (M_A)$; Activity $\frac{(M_A)\,\alpha_A}{M + (M_G) + (M_A)}$
Add K_2CO_3 and distill. Distillate richer in methanol.	$\text{Activity} = \frac{(M_G)\,\alpha_G}{M + (M_G) + (M_A)}$	$\text{Activity} = \frac{(M_A)\,\alpha_A}{M + (M_G) + (M_A)}$
Form p-nitrobenzoate ester, $C_8H_7O_4N$, burn to CO_2, and count activity	$a_1 = \frac{\text{Activity}}{8[M + (M_G) + (M_A)]} = \frac{(M_G)\,\alpha_G}{8[M + (M_G) + (M_A)]}$	$a_2 = \frac{\text{Activity}}{8[(M + (M_G) + (M_A)]} = \frac{(M_A)\,\alpha_A}{8[(M + (M_G) + (M_A)]}$
Simultaneous equations	$8a_1[M + (M_G) + (M_A)] = (M_G)\,\alpha_G$	$8a_2[M + (M_G) + (M_A)] = (M_A)\,\alpha_A$
Solve (where $A_1 = 8a_1$ and $A_2 = 8a_2$).	$(M_A) = \frac{A_2 M\,\alpha_G}{\alpha_A\alpha_G - A_2\alpha_G - A_1\alpha_A}$;	$(M_G) = \frac{A_1 M\,\alpha_A}{\alpha_A\alpha_G - A_2\alpha_G - A_1\alpha_A}$

[a]Except for the location of the carbon-14 label, the two runs are identical.

The method was tested using aqueous solutions with 1, 4, and 16 p.p.m. methanol(^{14}C). Twenty milliliter aliquots were taken from these solutions and subjected to the above ester synthesis. The product melting points were 94.0-94.5°C., 94.0-94.5°C., and 93.5-95.0°C., respectively. Literature values for the melting point of methyl p-nitrobenzoate are 95-96°C. (67), and 96°C. (68). The products were weighed on a semimicro balance into Van Slyke combustion chambers. The samples were burned and the activity of the resulting carbon dioxide was determined. The activity found was compared with the calculated activity. Table XXIII shows the results.

TABLE XXIII

TEST OF METHANOL ISOTOPE DILUTION ANALYSIS

Methanol(^{14}C), p.p.m.	Activity Found[a]	Average Activity Found[a]	Calculated Activity[a,b]	Error, %
1	62.4 ± 3.0 59.0 ± 3.4	60.7 ± 3.2	56.4	7.6 (high)
4	218 ± 5.0 213 ± 6.2	216 ± 5.6	225.2	4.1 (low)
16	769 ± 11.0 747 ± 9.0	758 ± 10	894.4	15.3 (low)

[a]Disintegrations per minute per milligram carbon in the methyl p-nitrobenzoate.

[b]Calculated from the dilution which the original 0.5 millicurie of carbon-14 methanol had undergone.

METHOD OF ISOTOPE DILUTION ANALYSIS FOR UNREACTED GLUCOSIDE

Removal of inorganic salts was the biggest problem in this analysis. Extraction, chromatography, and crystallization were used to remove these salts. These three techniques will be discussed below, and the order of their use in obtaining

pure samples of isotopically diluted MBG will be summarized in tables. After obtaining pure samples of the MBG, the samples were weighed into Van Slyke combustion chambers using a semimicro balance and their activity determined using the methods of Appendix II. The percentage of MBG which remained after the oxidation could then be calculated.

EXTRACTION

An aliquot of the oxidation solution was diluted with an accurately weighed amount of MBG. The solution was concentrated to dryness under reduced pressure. The solids were transferred to an extraction thimble which would fit in a small Soxhlet extractor. This extraction thimble was all glass, constructed by cutting the stem off of a coarse sintered glass microfilter. The extractor was assembled with 25 ml. of n-propanol in the Soxhlet flask. Extraction required 30 minutes to two hours, depending upon the drainage through the thimble. The extract was concentrated to dryness under reduced pressure.

During one extraction[1] the specific activity of the original solids, the extracted solids, and the residue remaining in the thimble was measured. These data were used for calculating the efficiency of these extractions. The results, given in Table XXIV, show that the extraction increases the ratio of carbohydrates to salts from about 1:20 to nearly 1:1.

[1] In this case, the aliquot of the oxidation solution (the 18-hr. oxidation solution from Oxidation 6) had not been diluted with unlabeled MBG.

TABLE XXIV

EFFICIENCY OF THE EXTRACTION PROCEDURE

Material	Weight, mg.	Carbohydrates, %
Original solids	613.5	5.74
Residue	537.5[a]	0.55
Extract	76.0[b]	42.48

[a]Calculated from the activity. The weighed amount of residue was 545.4 mg. The extra 7.9 mg. could easily have been water.

[b]By difference.

CHROMATOGRAPHY

Separation of MBG from the inorganic salts could also be accomplished chromatographically, provided the initial ratio of salt to carbohydrate was not too high. Too much salt would prevent proper development of the chromatograms. The extract from the Soxhlet extractors could be chromatographed successfully. Also, if a very large amount of MBG was added to the aliquot of the oxidation solution during the dilution, the sample could be chromatographed successfully.

Whatman number 17 chromatographic paper was used for these purifications. As much as 80 mg. of solids per inch could be streaked across the starting line on this very heavy paper. Solvents A and B, Appendix III, were used. After development, the band of MBG was found by spraying a guide strip with the silver nitrate spray described in Appendix III. The guide strip was either a narrow strip cut from the chromatogram, or a layer of fibers stripped from the dry chromatograms with transparent pressure-sensitive tape.

The band containing the MBG was cut from the chromatogram. The MBG was eluted from the paper with water. Darco G-60 carbon and Amberlite MB-3 monobed

ion-exchange resin were added to remove traces of color and the remaining ions. The carbon and resin were filtered out and the filtrate concentrated under reduced pressure.

CRYSTALLIZATION

Crystallization of the isotope dilution samples was made from 50% aqueous ethanol. Small crystallization dishes of about 0.25-ml. capacity were used. These dishes were made by cutting the bottom from a 4-ml. test tube and then attaching a glass rod (Fig. 24). A one-hole stopper was a suitable support. Crystallization was carried out in a desiccator over calcium chloride.

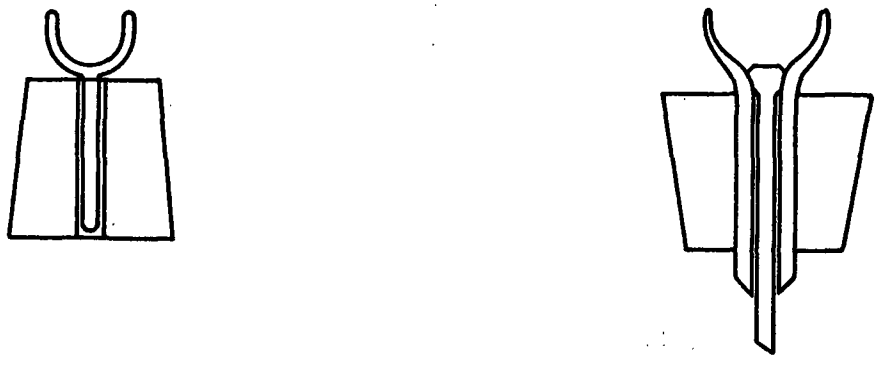

Figure 24. Microcrystallization Dish and Microfilter

Crystals were filtered from the mother liquor with the microfiltration apparatus shown in Fig. 24. The filter was made from capillary tubing and glass rod, using silicon carbide grinding compound to grind the joint between the two pieces. The mother liquor could pass through the ground glass joint, leaving the crystals behind.

ORDER OF USE OF THE PROCEDURES IN THE ANALYSES

The method of analysis was somewhat improved as this study progressed. Also, the earlier results determined, to some extent, the exact method of analysis in

some of the later determinations. For these reasons, the exact order in which the above techniques were applied varied for different samples. Tables XXV and XXVI summarize the order of use of the techniques, and the results.

METHOD OF ISOTOPE DILUTION ANALYSIS FOR FORMALDEHYDE AND FORMIC ACID

Parts of this method are similar to certain procedures in methods given by Sakami (69), Eisenberg (70), and Pirie (71).

A stock aqueous formaldehyde solution[1] was analyzed[2] for its formaldehyde content, which was found to be 36.88%. Its density was found to be 1.0886 g./ml. at 20°C. For isotope dilution, 6.8479 g. of this solution and 3.8247 g. of formic acid[3] were combined, and water was added to make 100 ml. at 20°C. One milliliter of this solution containing 10.10 mg. of carbon in the formaldehyde and 9.88 mg. of carbon in the formic acid was used in each analysis.

The formaldehyde and formic acid were separated, after isotope dilution of the sample solution, by distillation under reduced pressure. The formaldehyde was separated first, by distilling from the solution in which the pH had been adjusted to about 9.5; then the pH was readjusted to about 3, and the formic acid separated. The 25-ml. distilling flask was about three-fourths full of glass beads and boiling granules to stop violent bumping. Also, the neck of the distilling flask had been coated lightly with silicon stopcock grease to help stop foam

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[1] Mallinckrodt Chemical Works, Formaldehyde Solution, approximately 37%.

[2] TAPPI Method T 600 m-45.

[3] Matheson, Coleman, and Bell, 98-100% Formic Acid. In making the above solution, 99% purity was assumed.

TABLE XXV

PROCEDURES AND RESULTS FOR ISOTOPE DILUTION ANALYSIS OF UNREACTED GLUCOSIDE

Oxidation No.	Oxidation Time, hr.	Samples[a]	Procedure[b]	Results, % Unreacted MBG[c]	Results, % MBG Oxidized[d]
6	9	499, 500	A	87.1, 88.8	12.9, 11.2
		499, 500(P)	B	87.3, 87.8	12.7, 12.2
	18	439, 440	C	87.1, 90.8	12.9, 9.2
		441, 442	D	87.7, 88.5	12.3, 11.5
		455, 456	E	89.2, 89.4	10.8, 10.6
		455, 456(P)	F	85.8, 83.8	14.2, 16.2
7	9	495, 496	A	85.1, 85.2	14.9, 14.8
		495, 596(P)	B	84.4, 84.0	15.6, 16.0
	18	497, 498	A	83.6, 82.2	16.4, 17.8
		497, 498(P)	B	82.3, 82.7	17.7, 17.3
8	1.5	631, 632	G	86.9, 85.9	13.1, 14.1
		631, 632(P)	H	87.6, 89.7	12.4, 10.3
		654, 655	I	95.6, 91.1	4.4, 8.9
	4	633, 634	G	82.6, 89.8	17.4, 10.2
		633, 634(P)	H	90.8, 82.0	9.2, 18.0
		656, 657	I	93.0, 92.7	7.0, 7.3
	9	635, 636	G	80.2, 77.6	19.8, 22.4
		635, 636(P)	H	82.2, 88.1	17.8, 11.9
		658, 659	I	84.9, 87.1	15.1, 12.9
	18	637, 638	G	68.9, 77.3	31.1, 22.7
		637, 638(P)	H	74.6, 78.1	25.4, 21.9
		660, 661	I	80.7, 77.1	19.3, 22.9

[a] Original laboratory sample numbers. (P) refers to a repurification of the same sample, as explained in the procedures.

[b] The procedures referred to here are outlined in Table XXVI.

[c] The 90% confidence limits on the counting were in the range ± 0.5 - ± 1.5%.

[d] The results underlined were considered most reliable and were the ones shown in Fig. 13. The reasons for considering the underlined results more reliable are: Samples from Procedure B were taken in preference to those from Procedure A because they were the same dilution samples but purified one more time. The same reason holds for choosing "F" over "E", and, with one exception, for choosing "H" over "G". In that one case, some of the sample in "H" was probably lost during combustion. Results from Procedures C and D were not considered reliable because dilution was not the first step of the procedure. Most results for 1.5 hours' oxidation in oxidation eight were higher than those for 4-hr. oxidation. They were therefore not considered. The reason for this difficulty at 1.5 hours is not known.

TABLE XXVI

PROCEDURES FOR ISOTOPE DILUTION ANALYSIS OF UNREACTED GLUCOSIDE

A	B	C
Isotope dilute Extract carbohydrates Crystallize Recrystallize Determine specific activity	Combine the crystals and the mother liquor from recrystallization in A Purify chromatographically using Solvent A of Appendix III Crystallize Determine specific activity	Extract carbohydrates Isotope dilute Ion exchange using MB-3 resin Crystallize Recrystallize Determine specific activity

D	E	F
Extract carbohydrates Isotope dilute Crystallize Recrystallize Determine specific activity	Isotope dilute Extract carbohydrates Crystallize Determine specific activity	Chromatographically purify crystals from E in Solvent A of Appendix III Crystallize Determine specific activity

G	H	I
Isotope dilute Extract carbohydrates Chromatographically purify in Solvent A of Appendix III Crystallize Determine specific activity	Combine crystals and mother liquor from G Chromatographically purify in Solvent B of Appendix III Crystallize Determine specific activity	Isotope dilute with a large amount of MBG so the salt-to-carbohydrate ratio is small Chromatographically purify in Solvent A of Appendix III Chromatographically purify in Solvent B of Appendix III Crystallize Determine specific activity

before it reached the delivery tube of the flask. The flask was immersed in a mineral oil bath which could be heated. The distillates were collected in 50-ml. round-bottom flasks with one standard 19/38 ground glass neck. The receiving flasks were immersed in an ice-water bath. A rubber stopper held them on the distilling flask. Vacuum was drawn through a tube through the stopper.

In later steps of this procedure, the 50-ml. receiving flasks were stoppered with 19/38 ground glass connecting tubes, each of which had a stopcock. To this connecting tube, a joint which fitted on the Van Slyke apparatus[1] could be attached with a short section of heavy rubber tubing. Figure 25 shows this apparatus.

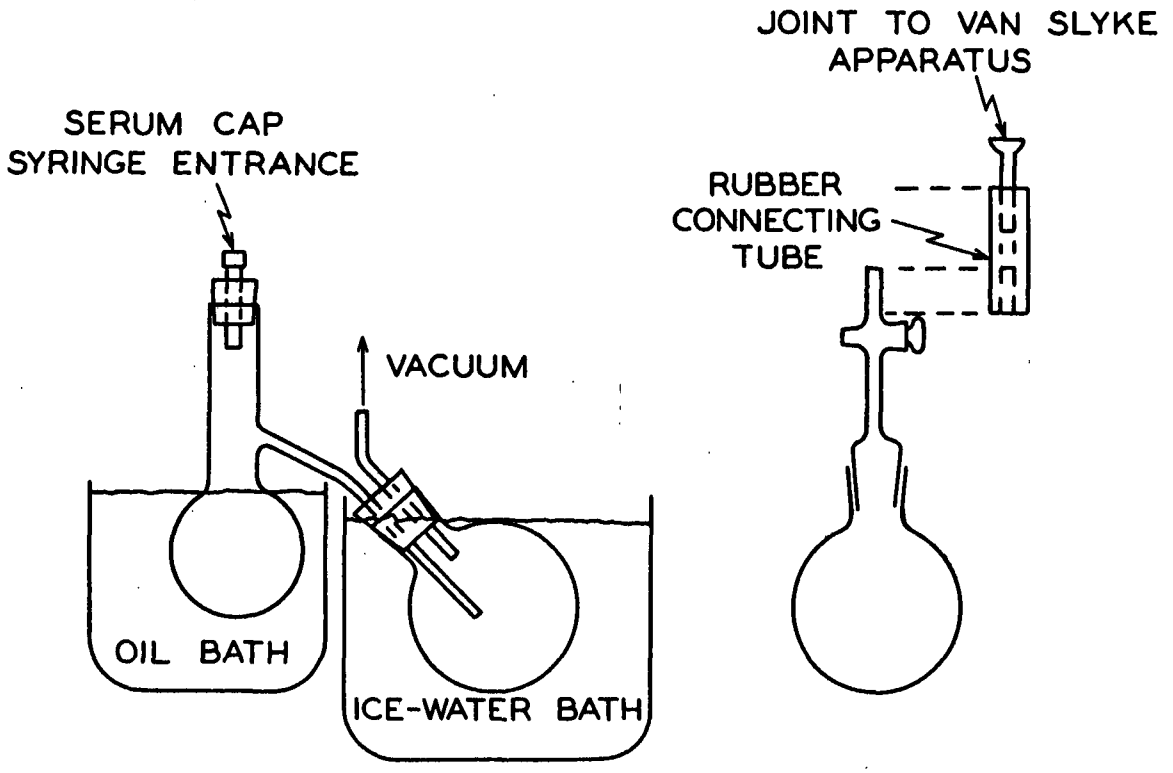

Figure 25. Apparatus Used in Formaldehyde and Formic Acid Determinations

[1]The receiving flask, with its connecting tube and stopcock, then replaced the reservoir D, and combustion tube A, on the Van Slyke apparatus, Fig. 18.

A mixed indicator was used in the distillation flask to aid in adjusting pH. A dilute aqueous solution of methyl yellow and thymolphthalein gave a red color when the pH was below approximately 3, yellow between pH 3 and approximately 9.5, and blue above 9.5.

Later in the procedure, a reagent described by Sakami (69) was used. It consisted of a carbon dioxide-free, aqueous solution containing 8% mercuric chloride, 2% sodium acetate[1], and 2% acetic acid. This reagent will oxidize formic acid to carbon dioxide, but will not oxidize formaldehyde.

The flow chart of Fig. 26 outlines the procedures for this analysis. The following paragraphs describe the steps of the procedure.

Step A. To the distillation flask is added 10 ml. of the oxidation solution and 25 drops of the mixed indicator. Isotope dilution is then made by adding, with agitation, 1 ml. of the formaldehyde-formic acid solution described on page 114.

Step B. Drops of 1N sodium hydroxide are added until the color indicates the pH is about 9.5. Then 10 more drops are added. This converts all formic acid to sodium formate, preventing it from distilling. A vacuum is applied (27-28 inches of Hg), and the solution is distilled to near dryness by heating slowly to 55-80°C. The vacuum is released and the oil bath cooled (or replaced with a cool one). Using a syringe, 5 ml. of distilled water are added. The vacuum is reapplied and the solution distilled again to near dryness. Water is added again, as before, and the distillation repeated a third time. The combined distillate containing formaldehyde, and probably enough methyl yellow to give it a yellow tint, is removed. New receiving flasks are placed on the apparatus.

Step C. The residue is cooled and 5 ml. of distilled water are added. Drops of 1N sulfuric acid are added until the color indicates the pH is approximately 3; then 5 more drops are added. This converts the sodium formate to formic acid so it may be distilled. Distillation is made twice, in the same manner as described in Step B. The receiving flasks containing the formic acid are removed. A trace of methyl red powder is added. The formic acid is neutralized by adding 1N sodium hydroxide.

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[1] $NaC_2H_3O_2 \cdot 3H_2O$.

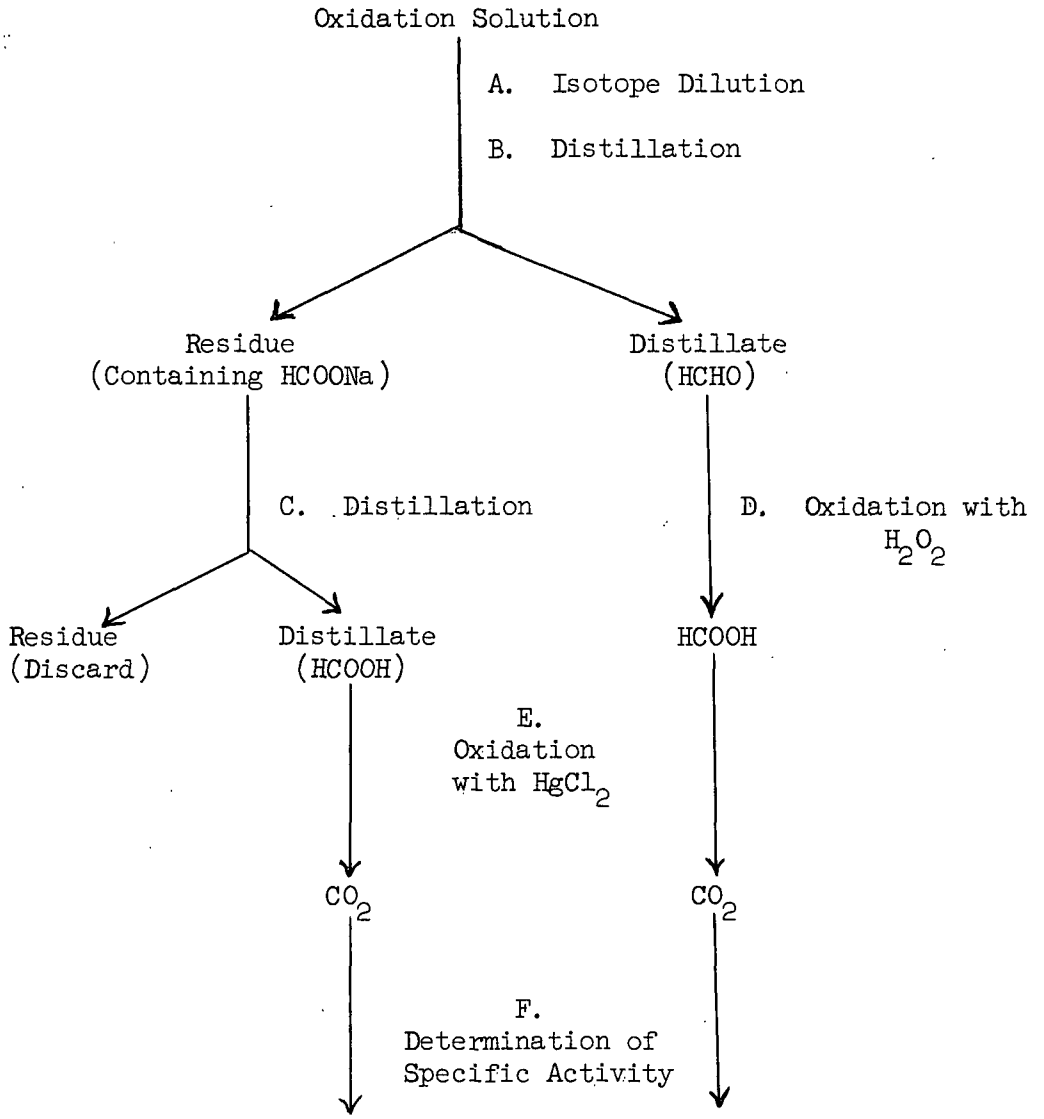

Figure 26. Outline of Formaldehyde and Formic Acid Analyses

Then the pH is more carefully adjusted by adding, first, drops of 1N sulfuric acid until the solution is red and, then, drops of 1N sodium hydroxide until test paper shows the pH to be about 11. The solution is then concentrated to about 0.5 ml. under reduced pressure.

Step D. A trace of methyl red powder, 4 ml. of 1N sodium hydroxide, and 3.4 ml. of 3% hydrogen peroxide are added to the formaldehyde distillate. It is heated 10 min. in a boiling water bath to oxidize the formaldehyde to sodium formate. One drop of dilute ferric nitrate is added, and the solution is heated an additional 10 min. to decompose any remaining peroxide. The solution is cooled, glacial acetic acid is added until the methyl red indicates an acidic pH, and then drops of 1N sodium hydroxide are added until test paper shows the pH is about 11. The solution is concentrated to about 0.5 ml. under reduced pressure.

Step E. Both the formaldehyde and the formic acid have been diluted, separated, and converted to sodium formate. Therefore, the remaining steps are identical for the two analyses.

Twenty-five milliliters of the mercuric chloride reagent previously described is added to the receiving flask containing the sodium formate. The connecting tube and stopcock shown in Fig. 25 are fitted to the flask using heavy vacuum grease on the half of the joint farthest from the flask; this will keep the grease away from the solution. The flask is connected to a vacuum line and agitated for one minute with the stopcock open, to remove air and dissolved carbon dioxide; the stopcock is then closed. The flask is heated to 97-100°C. for 30 min. in a boiling water bath. The sodium formate is oxidized to carbon dioxide by the mercuric ion.

Step F. While being careful not to allow air to enter the cooled flask, 0.25 ml. of 70% sulfuric acid is added through the stopcock. The rubber connecting tube and glass joint shown in Fig. 25 are then slipped on the tube from the flask. The flask is connected to the Van Slyke apparatus.

The carbon dioxide is then absorbed into the sodium hydroxide-hydrazine solution in the Van Slyke apparatus, released again with lactic acid, and its specific activity determined. The normal procedure given in Appendix II is used, with the following exceptions:

1. Because there is a rubber connecting tube, the receiving flask may be carefully agitated to aid in transferring the carbon dioxide from it into the sodium hydroxide – hydrazine solution.

2. There may be more carbon dioxide in the samples than can be used in the Bernstein-Ballentine tubes. After the carbon dioxide has been released with lactic acid, it is compressed to about 600 mm. Hg pressure and the volume noted. If the volume is more than about 4 ml., the excess is carefully released to the atmosphere (after further compressing it to above atmospheric pressure). Then the mercury is lowered, the solution stirred vigorously again to release the last

traces of carbon dioxide, and the normal Van Slyke procedure continued. It should be noted that it is important to get very accurate pressure, volume, and temperature readings in this case because there is no weighed sample from which the weight of carbon transferred to the counting tube can be calculated. The amount of carbon found from the Van Slyke calculations has to be used to obtain the specific activity.

In preliminary trials, it was found that methanol is not oxidized by hydrogen peroxide or by the mercuric chloride reagent, and that formaldehyde is not oxidized by the mercuric chloride reagent.

In some preliminary trials, the distillations were made at atmospheric pressure and at higher temperatures. Also, less care was taken in pH adjustment. In the first trial, using an oxidation solution, no formaldehyde was obtained, and the formic acid was yellow and smelled of chlorine dioxide. Proper pH adjustment gave distillation of the formaldehyde; proper pH adjustment and lower temperatures stopped the formation of chlorine dioxide.

METHOD OF ISOTOPE DILUTION ANALYSIS FOR HYDROLYZABLE METHANOL

Hydrolyzable methanol was determined by first hydrolyzing, and then redetermining the methanol in the solution by isotope dilution. From the result of this methanol determination, the free methanol already found and the methanol which is hydrolyzed from the unreacted M*BG were subtracted. The difference is the amount of aglucone carbon still attached as a methoxyl group to some oxidized residue from the glucosyl group.

The method given below is the one used on the oxidation solutions. Variations of this method were used in some of the tests. These will be discussed later.

The hydrolysis chambers used were 200-ml. round-bottom flasks to which a stopcock had been sealed. They were of the same construction as the oxidation solution sample flasks shown in Fig. 9, including the serum cap entrance for a syringe needle.

A methanol-water solution containing 2 ml. (1.584 g.) of pure methanol in 50 ml. of aqueous solution at 20°C. was prepared for use in the isotope dilution.

The hydrolysis chamber was evacuated, the stopcock closed, and the serum cap slipped over the end of the entrance. Except when inserting solutions with a syringe, the stopcock was kept closed throughout the procedure. The needle would go through the hole in the plug of the stopcock and reach the bottom of the flask.

Ten milliliters of the oxidation solution, 5 ml. of the methanol-water isotope dilution solution, and 10 ml. of 1N sulfurous acid were injected into the evacuated hydrolysis chamber. The flask was immersed in an ethylene glycol bath at 80°C. for one hour. Then 25 ml. of 4N hydrochloric acid was injected, making the solution 2N in hydrochloric acid. Hydrolysis at 80°C. continued for 24 hours[1].

After 24 hours, the hydrolysis chamber was cooled, the serum cap removed, the stopcock opened to relieve the vacuum, and 20 ml. of potassium carbonate solution (0.5 g./ml.) added with a syringe. The solution could then be stored in a refrigerator, still in the hydrolysis chambers.

Methanol from a 20-ml. sample of the neutralized hydrolysis solution was distilled off and converted to methyl p-nitrobenzoate, according to the method already described. Determination of the specific activity of this methyl p-nitrobenzoate, followed by the usual isotope dilution calculations, gave the results presented in Table XI in the Experimental section.

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[1] Hydrolysis of MBG should be 99.5% complete in six hours according to calculations made from rate constants given by Moelwyn-Hughes (72) and by Overend and co-workers (73).

In developing the method described above, certain preliminary runs were made. In the first five of these runs, a simulated oxidation solution was used. It had MBG and salt concentrations approximating those in the oxidation solutions, as shown in Table XXVII.

TABLE XXVII

CONCENTRATIONS IN THE SIMULATED OXIDATION SOLUTIONS

Material	Concentration, mg./ml.
M*BG	1.70
NaCl	14.4
$NaClO_3$	12.0
Na_2SO_4	2.0

In the first preliminary run, in which no sulfurous acid was used, available chlorine was produced after a few minutes' heating. It gave the solution a yellow color and caused the solution to give a positive test with starch-potassium iodide test paper. Because the available chlorine would have caused oxidation, the run was stopped. The available chlorine was probably chlorine dioxide, produced by the reaction:

$$NaClO_3 \xrightarrow[\text{organic reductant}]{H^+} ClO_2 \qquad (20).$$

In the second run, some sulfurous acid, to destroy available chlorine, was added along with the hydrochloric acid. Apparently, not enough was added because available chlorine was present for a short time (less than two hours). The results were numerically good, but were higher than those for later runs in which no available chlorine appeared. Some of the isotope dilution methanol may have been oxidized through Reaction (20), with the methanol serving as the reductant.

This reaction occurs in the Solvay process for making chlorine dioxide (66). If methanol was destroyed, the results were too high.

In the third, fourth, and fifth runs, the method was the one given above for the oxidation solutions, except for the use of the simulated oxidation solution instead of an actual one, and varying hydrolysis times. Extra hydrolysis time did not improve the results. The average result for these runs was 92.25%. There may have been some reverse reaction:

$$CH_3OH + \text{glucose} \longrightarrow \text{MBG} \qquad (6).$$

Incomplete chromatographic evidence for MBG was found in deionized solutions from some of the runs, but none was isolated for specific activity determinations.

In the sixth run, no salts or sulfurous acid were used. Other concentrations were unchanged. Within experimental accuracy, all the aglucone carbon was accounted for.

In the seventh run, the MBG was replaced by enough methanol and glucose to represent completely hydrolyzed MBG. The salt concentrations were unchanged, and sulfurous acid was used. The results for duplicate runs did not agree.

Table XXVIII shows the results for these preliminary runs.

TABLE XXVIII

RESULTS OF PRELIMINARY HYDROLYSIS RUNS

Run	Material Used	1N H_2SO_3, ml.	Hydrolysis Time, hr.	Aglucone Carbon Found, %
1	Simulated oxidation solution	0	--	--
2	Simulated oxidation solution	2	24	101.1, 99.4
3	Simulated oxidation solution	10	24	91.7, 91.6 [b]
4	Simulated oxidation solution	10	48	94.8, -- [b]
5	Simulated oxidation solution	10	72	90.9, -- [b]
6	M*BG only	0[a]	24	102.6, 102.2
7	Methanol + glucose + salts	10	24	95.8, 107.4

[a] Ten milliliters of distilled water were added instead, to keep other concentrations the same as in other runs.

[b] Average 92.25%.

APPENDIX VII

COMPARISON OF RATE CONSTANTS

The rate of oxidant consumption found in this work was compared with the rate of consumption calculated from Grillo's rate and catalytic constants (9). In this discussion the rate refers to the rate of oxidant consumption due to oxidation of the carbohydrates present. A separate correction was made for the disproportionation of oxidant into chloride and chlorate.

The rate of oxidant consumption may be expressed as:

$$-\frac{d(Ox)}{dt} = k^{obs}(Ox)(MBG)$$

$$= k^{Cl_2}(Cl_2)(MBG) + k^{HOCl}(HOCl)(MBG)$$

$$+ k^{Cl_2}_{Cl^-}(Cl_2)(MBG)(Cl^-) + k^{HOCl}_{Cl^-}(HOCl)(MBG)(Cl^-)$$

$$+ k^{Cl_2}_{OCl^-}(Cl_2)(MBG)(OCl^-) + k^{HOCl}_{OCl^-}(HOCl)(MBG)(OCl^-)$$

$$+ k^{Cl_2}_{OH^-}(Cl_2)(MBG)(OH^-) + k^{HOCl}_{OH^-}(HOCl)(MBG)(OH^-) \qquad (21),$$

where;

(Ox) = concentration of available chlorine, moles/liter

t = oxidation time, sec.

k^{obs} = second-order rate constant for the observed consumption of oxidant, liters/mole sec. (22)

$( )$ = concentration, moles/liter

k^{Cl_2} = rate constant for oxidation by Cl_2, liters/mole sec. (23)

k^{HOCl} = rate constant for oxidation by HOCl, liters/mole sec. (24)

$k^{Cl_2}_{Cl^-}$ = catalytic constant for catalysis of Cl_2, oxidation by Cl^-, $(liters)^2/(mole)^2$ sec. (25)

$k^{HOCl}_{Cl^-}$ = catalytic constant for catalysis of HOCl oxidation by Cl^-, $(liters)^2/(mole)^2$ sec. (26)

$k^{Cl_2}_{OCl^-}$ = catalytic constant for catalysis of Cl_2 oxidation by OCl^-, $(liters)^2/(mole)^2$ sec. (27)

$k^{HOCl}_{OCl^-}$ = catalytic constant for catalysis of HOCl oxidation by OCl^-, $(liters)^2/(mole)^2$ sec. (28)

$k^{Cl_2}_{OH^-}$ = catalytic constant for catalysis of Cl_2 oxidation by OH^-, $(liters)^2/(mole)^2$ sec. (29)

and $k^{HOCl}_{OH^-}$ = catalytic constant for catalysis of HOCl oxidation by OH^-, $(liters)^2/(mole)^2$ sec. (30).

Grillo included other terms containing catalytic constants for ionic species from his buffers, such as $k^{HOCl}_{OAc^-}$, but these terms are of no interest in this study.

Grillo has evaluated rate and catalytic constants (23), (24), (25), (26), (28), and (30) using certain assumptions about the activity coefficients in his solutions. He found catalytic constant (30) is very nearly zero. Making the reasonable assumption that the terms involving constants (27) and (29) are negligible at pH 7, and dividing (21) by (MBG), one obtains:

$$k^{obs}(Ox) = k^{Cl_2}(Cl_2) + k^{HOCl}(HOCl) + k^{Cl_2}_{Cl^-}(Cl_2)(Cl^-) + k^{HOCl}_{Cl^-}(HOCl)(Cl^-) + k^{HOCl}_{OCl^-}(HOCl)(OCl^-) \quad (31).$$

Upon substitution of Grillo's values, this equation becomes:

$$k^{obs}(Ox) = 4.4(10^{-4})(Cl_2) + 4.0(10^{-4})(HOCl) + 7.0(10^{-4})(Cl_2)(Cl^-) - 3.0(10^{-4})(HOCl)(Cl^-) + 0.29(HOCl)(OCl^-) \quad (32).$$

The initial conditions for oxidation eight in this study were[1]:

$$(Ox) = 0.0247 \text{ mole/liter},$$
$$(Cl_2) = 8.9(10^{-7}) \text{ mole/liter},$$
$$(HOCl) = 0.0178 \text{ mole/liter},$$
$$(OCl^-) = 0.0069 \text{ mole/liter},$$
$$(Cl^-) = 0.236 \text{ mole/liter, and}$$
$$(MBG) = 0.01006 \text{ mole/liter.}$$

Upon substituting these initial conditions in the above equation, one obtains:

$$k^{obs} = (0.00016 + 2.88 + 0.00006 - 0.51 + 14.4)(10^{-4}) \quad (33)$$

or

$$k^{obs} = 16.8(10^{-4}) \text{ liters/mole sec.} \quad (34)$$

The rate equation calculated from Grillo's constants is, therefore:

$$-\frac{d(Ox)}{dt} = 16.8(10^{-4})(MBG)(Ox) \quad (9).$$

The terms in Equation (33) represent, in order, oxidant consumption due to oxidation by Cl_2, due to oxidation by HOCl, due to chloride ion-catalyzed oxidation by Cl_2, due to chloride ion inhibition of oxidation by HOCl, and due to hypochlorite ion-catalyzed oxidation by HOCl. The terms involving molecular chlorine are insignificant in comparison with the terms involving hypochlorous acid.

The rate constant $\underline{k}^{obs}$ for the three main oxidations in this study were calculated using the equation:

---

[1]Calculated from the data in Appendix IX; the hydrolysis constant of chlorine at 25°C.: $3.9(10^{-4})$; the ionization constant of hypochlorous acid at 25°C.: $3.2(10^{-8})$; and Grillo's assumptions regarding activity coefficients.

$$-\frac{d(Ox)}{dt} = k^{obs}(MBG)(Ox) \qquad (35).$$

Values of $-d(Ox)/dt$ were obtained from the initial slopes of the curves in Fig. 5, 6, and 7, showing consumption of oxidant versus time of oxidation. These data, together with the initial concentrations of oxidant and glucoside are given in Table XXIX.

TABLE XXIX

INITIAL CONCENTRATIONS AND RATES OF OXIDANT CONSUMPTION DURING OXIDATIONS

Oxidation	Initial Oxidant Consumption		Concentration	
	$\frac{\text{meq.}}{\text{liter hour}}$	$\frac{\text{mole}}{\text{liter second}}$	Oxidant $\frac{\text{mole}}{\text{liter}}$	Glucoside $\frac{\text{mole}}{\text{liter}}$
6	$\frac{0.005}{9} = 7.73(10^{-8})$		0.0246	0.01005
7	$\frac{0.0010}{2} = 6.95(10^{-8})$		0.0233	0.00965
8	$\frac{0.0018}{3} = 8.33(10^{-8})$		0.0247	0.01006

The calculated k^{obs} values for Oxidations 6, 7, and 8 are therefore $3.12(10^{-4})$, $3.09(10^{-4})$, and $3.35(10^{-4})$ liters/mole second, respectively. The average for duplicate Oxidations 6 and 8 is $3.24(10^{-4})$ liters/mole second, giving rate equation:

$$-\frac{d(Ox)}{dt} = 3.24(10^{-4})(MBG)(Ox) \qquad (8).$$

The ratio of the k^{obs} value in Equation (9) obtained using Grillo's constants to the k^{obs} value in Equation (8) obtained entirely from data from this study is 5.2 to 1.0. One would expect a ratio of about 2.0 to 1.0 because Grillo's constants were obtained at 35.7°C. instead of at 25°C. as used in this study. The agreement between the rate constants from the two studies is therefore quite good considering the experimental errors and the assumptions necessary for the calculations.

APPENDIX VIII

PROCEDURE FOR FRACTIONATING THE OXIDATION SOLUTIONS

This procedure was used by Henderson (8), and is best explained in the form of a flow chart. It was used for some of the preliminary oxidations, but was not used for the main oxidations in this study. In the fractionations made, difficulty with salts coming through the procedure was encountered. This was particularly true for ammonium sulfate. Optimum volumes and concentrations for the various solutions were never worked out. Figure 27 shows the method.

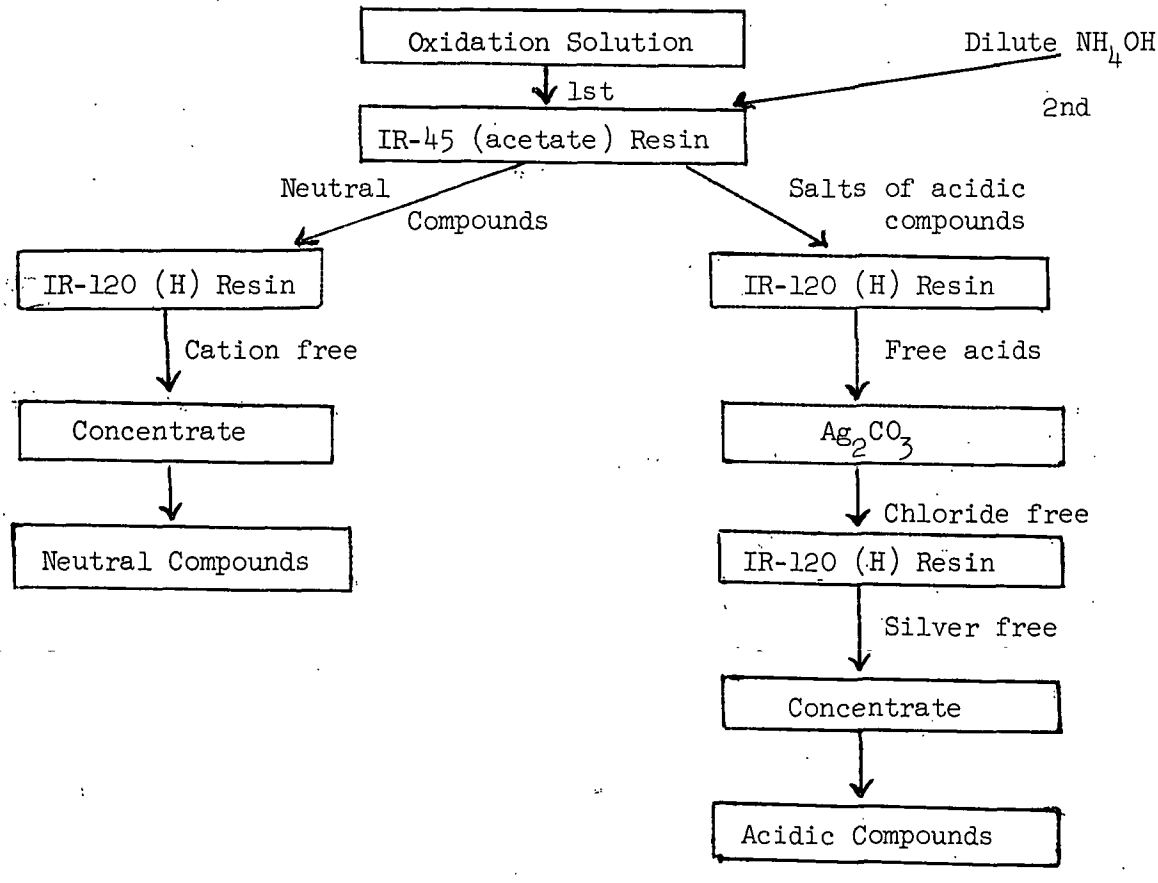

Figure 27. Procedure for Fractionating the Oxidation Solutions

APPENDIX IX

DATA

The data in this appendix are arranged to correspond to the order in the main body and the other appendices of this paper. Before each table or paragraph, the page to which it corresponds is given; a short identifying phrase or title will also be given.

Page 22, Figure 4

TABLE XXX

EQUILIBRATION OF AQUEOUS CHLORINE PRIOR TO OXIDATION FIVE

Equilibration Time, hr.:min.	Titrations		
	Sample, ml.	Thio., ml. 0.0971N	Available Cl, N
a			0.186
0:00	2.0	3.80	0.184
0:15	2.0	3.65	0.177
0:30	2.0	3.55	0.172
10:15	2.0	1.98	0.096
12:15	5.0	4.48	0.087
16:15	5.0	4.04	0.078
18:15	5.0	3.83	0.074
20:30	5.0	3.65	0.071

[a]Calculated from the dilution of the stock solution.

During the equilibration, 16 ml. of 1.0N sodium hydroxide solution had been added for pH control. To the 494 ml. of equilibrated solution, 50 ml. of solution containing 0.6806 g. of anhydrous MBG* was added after 21 hours 15 minutes equilibration. The oxidation is described on page 97.

Page 22, Figure 4

TABLE XXXI

EQUILIBRATION OF AQUEOUS CHLORINE PRIOR TO OXIDATION SIX

Equilib. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations: Sample, ml.	Titrations: Thio., ml. 0.0968N	Titrations: Avail. Cl, N
a			580.6			0.439
0:00		6.0[b]	574.6	5.0	14.28	0.276
0:25	9.7[c]		584.3			
0:30		7.0[b]	577.3	5.0	11.79	0.232
1:00		6.5[b]	570.8	5.0	10.84	0.210
1:40	4.9[c]		575.7			
2:00		7.0[b]	568.7	5.0	9.06	0.175
	12.1[c]		580.8			
10:40		6.0[b]	574.8	5.0	5.11	0.099
12:00		11.0[b]	563.8	10.0	9.31	0.090
	1.7[c]		565.5			
15:00		11.5[b]	554.0	10.0	8.32	0.0805
	2.0		556.0			
19:20		11.0[b]	545.0	10.0	7.50	0.0726
24:00		11.0[b]	534.0	10.0	6.81	0.0659
33:30		11.0[b]	523.0	10.0	5.77	0.0559
39:35		11.0[b]	512.0	10.0	5.24	0.0507
39:45		25.0[d]	487.0			
40:00	13.0[e]		500.0			0.0491[f]

[a] Calculated normality from the dilution of the chlorine stock solution.

[b] Sample removed by syringe for titration of the available chlorine.

[c] 1.0N NaOH added by the automatic pH control apparatus.

[d] Sample removed by syringe and placed in an opaque flask in the 25°C. water bath. Titrations of this sample gave the strength of the chlorine solution in the absence of MBG*.

[e] 0.9758 g. (5.025mM) of anhydrous MBG* dissolved in 13 ml. of water was injected by syringe.

[f] Calculated from the dilution caused by the MBG* solution. Without this dilution the solution would have been 0.0504N in available chlorine at 40 hr.

Page 22, Figure 4

TABLE XXXII

EQUILIBRATION OF AQUEOUS CHLORINE PRIOR TO OXIDATION SEVEN

Equilib. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations		
				Sample, ml.	Thio., ml. 0.0967N	Avail. Cl, N
a			580.0			0.443
0:00		6.5[b]	573.5	5.0	14.42	0.279
0:20	8.1[c]		581.6			
0:30		7.0[b]	574.6	5.0	12.35	0.239
1:00	5.3[c]	7.0[b]	572.9	5.0	10.97	0.212
1:40	2.9[c]		575.8			
2:00	2.3[c]	6.0[b]	572.1	5.0	9.14	0.177
	10.3[c]		582.4			
11:15		13.5[b]	568.9	10.0	9.12	0.088
11:45	1.9[c]		570.8			
12:45	1.9[c]		572.7			
14:00		12.0[b]	560.7	10.0	8.25	0.080
17:00		12.5[b]	548.2	10.0	7.64	0.074
	1.7[c]		549.9			
20:00		12.2[b]	537.7	10.0	7.02	0.068
24:10		12.5[b]	525.2	10.0	6.47	0.063
34:45		12.7[b]	512.5	10.0	5.44	0.053
39:30		12.5[b]	500.0	10.0	5.02	0.0485
39:40		24.8[d]	475.2			
40:00	16.0[e]		491.2			0.0466[f]

[a]Calculated normality from the dilution of the chlorine stock solution.

[b]Sample removed by syringe for titration of the available chlorine.

[c]1.0N NaOH added by the automatic pH control apparatus.

[d]Sample removed by syringe and placed in an opaque flask in the 25°C. water bath. Titrations of this sample gave the strength of the chlorine solution in the absence of M*BG.

[e]0.9206 g. (4.740mM) of anhydrous M*BG dissolved in 16 ml. of water was injected by syringe.

[f]Calculated from the dilution caused by the glucoside solution. Without this dilution the solution would have been 0.0483N in available chlorine at 40 hr.

Page 22, Figure 4

TABLE XXXIII

EQUILIBRATION OF AQUEOUS CHLORINE PRIOR TO OXIDATION EIGHT

Equilib. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations: Sample, ml.	Titrations: Thio., ml., 0.0964N	Titrations: Avail. Cl, N
	[a]		1750			0.445
0:00		6.0[b]	1744	5.0	16.55	0.319
0:12	20.0[c]		1764			
0:17	20.0[c]		1784			
0:30		6.0[b]	1778	5.0	13.30	0.256
0:50	20.5[c]		1798.5			
1:00		6.0[b]	1792.5	5.0	11.36	0.219
1:25	14.5[c]		1807			
2:00		6.0[b]	1801	5.0	9.19	0.177
2:06	15.0[c]		1816			
3:00		6.0[b]	1810	5.0	7.85	0.151
3:05	12.5[c]		1822.5			
	28.5[c]		1851			
9:20		11.0[b]	1840	10.0	9.50	0.0916
	7.0[c]		1847			
12:00		11.0[b]	1836	10.0	8.45	0.0815
14:00		11.0[b]	1825	10.0	7.83	0.0755
	4.0[c]		1829			
16:00		11.0[b]	1818	10.0	7.37	0.0710
	6.0[c]		1824			
20:35		11.0[b]	1813	10.0	6.52	0.0629
21:15	5.0[c]		1818			
22:30		11.0[b]	1807	10.0	6.25	0.0603
	5.0[c]		1812			
30:10		11.0[b]	1801	10.0	5.40	0.0521
32:00		11.0[b]	1790	10.0	5.26	0.0507
32:15		11.0[b]	1779	10.0	5.24	0.0505
32:32		299.0[d]	1480			
32:50	20.0[e]		1500			0.0493[f]

[a] Calculated normality from the dilution of the chlorine stock solution.

[b] Sample removed by syringe for titration of the available chlorine.

[c] 1.0N NaOH added by the automatic pH control apparatus.

[d] Sample removed by syringe and placed in an opaque flask in the 25°C. water bath. Titrations of this sample gave the strength of the chlorine solution in the absence of M*BG.

[e] 2.9306 g. (15.092mM) of anhydrous M*BG dissolved in 20 ml. of water was injected by syringe.

[f] Calculated from the dilution caused by the M*BG solution. Without this dilution the solution would have been 0.500N in available chlorine at 32 hours 50 minutes.

Page 23, Figure 5

TABLE XXXIV

AVAILABLE CHLORINE CONCENTRATION DURING OXIDATION SIX

Oxid. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations: Sample, ml.	Titrations: Thio., ml., 0.0968N	Titrations: Avail. Cl, N	Corrected Blank[a], N
0.00[b]			500.0			0.0491[c]	0.0491
0:05		6.0[d]	494.0	5.0	2.56	0.0496	
0:30		6.0[d]	488.0	5.0	2.52	0.0488	
1:00		6.0[d]	482.0	5.0	2.46	0.0476	
1:30	0.65[e]		482.7				
2:00		6.0[d]	476.7	5.0	2.42	0.0469	
3:45		6.0[d]	470.7	5.0	2.33	0.0451	
3:46	0.88[e]		471.6				
7:45	1.02[e]		472.6				
8:40[f]				9.0	4.14	0.0445	0.0431
8:50		11.0	461.6	10.0	3.96	0.0383	
9:00		100.0[g]	361.6				
14:10	0.45[e]		362.0				
17:35[f]				10.0	4.22	0.0408	0.0395
17:45		11.0[d]	351.0	10.0	3.07	0.0297	
18:00	6.2[h]		357.2				
	17.1[j]		374.3				

[a] This is the normality in the blank solution adjusted to correspond to the normality in the oxidation solution by taking into consideration the dilution which occurred in the oxidation solution.

[b] MBG* was injected after 40 hours' equilibration of the chlorine oxidation solution (Footnote [e], Table XXXI).

[c] Calculated normality (Footnote [f], Table XXXI).

[d] Sample removed by syringe for titration of the available chlorine.

[e] 1.0N NaOH added by the automatic pH control apparatus.

[f] Titration of an aliquot of the blank solution.

[g] The nine-hour oxidation sample was removed by syringe. To it was added 2.1 ml. 2.03N H_2SO_3 to stop the oxidation, 5.5 ml. 1.0N NaOH and 0.2 ml. 1.0N H_2SO_4 to adjust the pH to approximately six. The total volume was then 107.8 ml. The sample was stored in a refrigerator.

[h] 2.03N H_2SO_3 was added to stop the oxidation. Starch-potassium iodide test paper was used to test for complete reduction of the available chlorine.

[j] 1.0N NaOH was added to bring the pH to 6.9. The solution was stored in a refrigerator.

Page 24, Figure 6

TABLE XXXV

AVAILABLE CHLORINE CONCENTRATION DURING OXIDATION SEVEN

Oxid. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations: Sample, ml.	Titrations: Thio., ml., 0.0967N	Titrations: Avail. Cl, N	Corrected Blank,[a] N
0:00[b]			491.2			0.0466[c]	0.0466
0:12		11.6[d]	479.6	10.0	4.81	0.0465	
0:25	0.9[e]		480.5				
	1.0[e]		481.5				
8:30[f]				10.0	4.49	0.0434	0.0418
8:45		12.3[d]	469.2	10.0	3.88	0.0375	
9:00		100.0[g]	369.2				
10:05	0.5[e]		369.7				
	0.7[e]		370.4				
17:30[f]				10.0	4.05	0.0392	0.0377
17:50		12.4[d]	358.0	10.0	3.12	0.0302	
18:00	12.0[h]		370.0				
	16.2[j]		386.2				
	1.0[k]		387.2				
	0.02[m]		387.2				
	0.05[n]		387.3				
	3.58[p]		390.9				

[a,d,e,f] Footnotes, Table XXXIV also apply to this table.

[b] M*BG was injected after 40 hours' equilibration of the chlorine oxidation solution (Footnote [e], Table XXXII).

[c] Calculated normality (Footnote [f], Table XXXII).

[g] The nine-hour oxidation sample (93.8 ml. of the solution and 123.4 ml. of the vapor; 20% of the contents of the oxidation flask) was removed by syringe and injected into an evacuated 250-ml. flask. 4.12 ml. of 0.9N H_2SO_3 was added to stop the oxidation. 4.6 ml. of 1.0N NaOH was added to adjust the pH to seven. 0.938 ml. of methanol-water solution (42 ml. methanol/200 ml. solution) was added for isotope dilution of the methanol. The sample volume was 103.46 ml. It was stored in a refrigerator.

[h] 0.9N H_2SO_3 was added to stop the oxidation.

[j] 1.0N NaOH was added to bring the pH to seven.

[k] 1.0 ml. water was added to rinse out the tygon tube from the control apparatus.

[m] 1.0N H_2SO_4, and

[n] 1.0N NaOH was added to adjust the pH to seven.

[p] Methanol-water solution (42 ml. methanol/200 ml. solution) was added for isotope dilution of the methanol.

Page 25, Figure 7

TABLE XXXVI

AVAILABLE CHLORINE CONCENTRATION DURING OXIDATION EIGHT

Oxid. Time, hr.:min.	Volume Added, ml. from	Volume Removed, ml. for	Total Volume, ml.	Titrations: Sample, ml.	Titrations: Thio., ml., 0.0964N	Titrations: Avail. Cl, N	Corrected Blank,[a] N
[b]			1500			0.0493[c]	0.0493
0:08		11.0[d]	1489	10.0	5.10	0.0492	
0:22[e]				10.0	5.16	0.0497	0.0490
1:00		11.0[d]	1478	10.0	4.99	0.0481	
1:08[e]				10.0	5.08	0.0490	0.0483
1:24 to 1:40		250.0[g]	1228				
2:30		11.0[d]	1219	10.0	4.74	0.0457	
2:39[e]				10.0	4.96	0.0478	0.0472
2:45	4.0[f]		1223				
3:30		11.0[d]	1212	10.0	4.60	0.0443	
3:39[e]				10.0	4.87	0.0469	0.0461
3:54 to 4:09		250.0[h]	962				
5:00		11.0[d]	951	10.0	4.40	0.0424	
5:10[e]				10.0	4.74	0.0457	0.0449
5:20[e]				10.0	4.74	0.0457	0.0449
6:30		11.0[d]	940	10.0	4.21	0.0406	
6:39[e]				10.0	4.67	0.0450	0.0443
8:00		11.0[d]	929	10.0	4.03	0.0388	
8:07[e]				10.0	4.59	0.0442	0.0435
8:52 to 9:08		180.0[j]	749				
9:35	2.0[f]		751				
10:00		11.0[d]	740	10.0	3.76	0.0362	
10:07[e]				10.0	4.45	0.0429	0.0421
10:20	1.0[f]		741				
12:00		11.0	730	10.0	3.55	0.0342	
12:08[e]				10.0	4.35	0.0419	0.0410
14:05	1.0[f]		731				
14:30		11.0[d]	720	10.0	3.31	0.0319	
14:38[e]				10.0	4.23	0.0408	0.0399
16:00		11.0[d]	709	10.0	3.15	0.0304	
16:08[e]				10.0	4.15	0.0400	0.0391
17:30		11.0[d]	698	10.0	3.00	0.0289	
17:37[e]				10.0	4.08	0.0393	0.0384
18:00	20.0[k]		718				
	4.5[m]		722.5				
	7.5[n]		730.0				
	0.2[p]		730.2				

Notes on next page.

Notes, Table XXXVI

[a]This is the normality in the blank solution adjusted to correspond to the normality in the oxidation solution by taking into consideration the dilution which occurred in the oxidation solution.

[b]M*BG was injected after 32 hours 50 minutes equilibration of the chlorine oxidation solution (Footnote [e], Table XXXIII).

[c]Calculated normality (Footnote [f], Table XXXIII).

[d]Sample removed by syringe for titration of the available chlorine.

[e]Titration of an aliquot of the blank solution.

[f]1.0N NaOH added by the automatic pH control apparatus.

[g]The 1.5-hour oxidation sample was removed by syringe and injected into a 509.8-ml. evacuated flask. Alternate samples of liquid and vapor were removed

Liquid	Vapor	Then the following were added by syringe:
60.0 ml.	27.4 ml.	10.75 ml. 2.1N H_2SO_3 to stop the reaction.
60.0	31.1	32.0 ml. 1.0N NaOH, and
60.0	35.1	1.45 ml. 2.0N HCl, and
40.0	26.3	0.2 ml. 1.0N NaOH to adjust the pH to 7.5.
30.0	21.3	
250.0		Total liquid volume: 294.4 ml. The sample was stored in a refrigerator.

[h]The 4.0-hour oxidation solution was removed and injected into a 520.0 ml. evacuated flask.

Liquid	Vapor	Then the following were added by syringe:
60.0 ml.	48.1 ml.	13.35 ml. 2.1N H_2SO_3 to stop the reaction,
60.0	53.8	34.2 ml. 1.0N NaOH to adjust the pH to 7.5.
60.0	60.1	
40.0	44.8	Total liquid volume: 297.6 ml. The sample
30.0	36.2	was stored in a refrigerator.
250.0		

[j]The 9.0-hour oxidation solution was removed and injected into a 507.4-ml. evacuated flask.

Liquid	Vapor	Then the following were added by syringe:
60.0 ml.	82.6 ml.	8.0 ml. 2.1N H_2SO_3 to stop the reaction,
60.0	92.5	2.1 ml. 10.0N NaOH, and
60.0	104.0	0.7 ml. 2.1N HCl to adjust the pH to 7.5.
180.0		Total liquid volume 190.8 ml. The sample was stored in a refrigerator.

[k]2.1N H_2SO_3 was added to stop the reaction.

[m]9.91N NaOH, and

[n]1.0N NaOH, and

[p]2.1N H_2SO_3 added to adjust the pH to 7.5.

Page 30, Table III

TABLE XXXVII

SPECIFIC ACTIVITY OF MBG*

Material	Dilution with MBG	Glucosyl Carbon, mg.	Net Counts per Min.	$\frac{V}{r}\frac{E}{}$	Specific Activity[a]
Crude MBG*	1:10	0.78	945	0.845	15,760
Recrystd. MBG*	1:10	0.78	942	0.845	15,720
Purified MBG*	1:10	0.814	962.5	0.8206	15,800

[a] dis./(min.)(mg. glucosyl C).

Page 31, Footnote 1

TABLE XXXVIII

CALCULATION OF SOLIDS CONTENT, OXIDATION SEVEN[a]

Time, hr.:min.	Added or Removed	Fraction of Solution	NaOH, ±	NaOH, Total, g.	Chlorine, ±	Chlorine, Total, g.	M*BG, ±	M*BG, Total, mg.
[b]				6.20		9.14		
[c]			+3.13	9.33				
0:00[d]	-6.5/580[e]	0.0112	-0.10	9.23	-0.10	9.04		
0:20	+8.1(0.04)[f]		+0.32	9.55				
0:30	-7.0/581.6	0.0120	-0.12	9.43	-0.11	8.93		
1:00	+5.3(0.04)		+0.21	9.64				
1:00	-7.0/(574.6+5.3)	0.0121	-0.12	9.52	-0.11	8.82		
1:40	+2.9(0.04)		+0.12	9.64				
2:00	+2.3(0.04)		+0.09	9.73				
2:00	-6.0/(575.8+2.3)	0.0104	-0.10	9.63	-0.09	8.73		
	+10.3(0.04)		+0.41	10.04				
11:15	-13.5/582.4	0.0232	-0.23	9.81	-0.20	8.53		
12:45	+3.8(0.04)		+0.15	9.96				
17:00	-24.5/572.7	0.0428	-0.43	9.53	-0.36	8.17		
	+1.7(0.04)		+0.07	9.60				
39:40	-74.7/549.9	0.1360	-1.30	8.30	-1.11	7.06		
0:00[g]							+920.6	920.6
0:12	-11.6/491.2	0.0236	-0.20	8.10	-0.17	6.89	-21.7	898.9
0:25	+1.9(0.04)		+0.08	8.18				
8:45	-12.3/481.5	0.0255	-0.21	7.97	-0.19	6.70	-22.9	876.0
9:00	-100.0/469.2	0.2132	-1.70	6.27	-1.43	5.27	-187.0	689.0
10:05	+1.2(0.04)		+0.05	6.32				
17:50	-12.4/370.4	0.0335	-0.21	6.11	-0.18	5.09	-23.0	666.0
18:00				6.11		5.09		666.0

Notes on next page.

Notes, Table XXXVIII

[a]Using data of Table XXXII and Table XXXV.

[b]Aliquot of chlorine stock solution, 0.78N in available chlorine, 330 ml., 35.5 g./eq. Starting solution was sodium hydroxide, 15 g./800 ml., chlorine was added, and 330 ml. were used.

[c]Adjustment of pH with 78.2 ml. 1.0N NaOH.

[d]Equilibration time for the chlorine solution.

[e]Removed 6.5 ml. of the 580 ml. for titration, or 1.12% of the solution.

[f]Added 8.1 ml. of 1.0N NaOH for pH control.

[g]Oxidation time for the glucoside.

It is possible, using data from Tables XXXII, XXXV, and XXXVII, to calculate what salts are present in the oxidation solutions after the oxidation and after the residual chlorine is reduced with sulfurous acid. Such a calculation for the eighteen-hour oxidation solution follows.

Total chlorine present after 18 hours	5.09 g.	
Available chlorine, 18 hours (0.0302N)(358 ml.)(0.0355 g./meq.)	0.38	
Chlorine which is not available chlorine	4.71 g.	
Assuming the only reaction is: $3OCl^- \longrightarrow ClO_3^- + 2Cl^-$		
Chloride present is (2/3)(4.71)	3.14 g.	
Chlorine in chlorate is (1/3)(4.71)	1.57	
H_2SO_3 reduced the available chlorine to chloride		
Total chloride is 3.14 + 0.38		3.52 g.
Chlorate is (83.5/35.5)		3.69 g.
The 10.8 meq. H_2SO_3 was converted to sulfate (10.8 meq.)(0.048 g. meq.)	0.52 g.	
H_2SO_4 was used to adjust the pH (0.02 ml.)(1.0N)	trace	
Total sulfate		0.52 g.
Sodium hydroxide present after 18 hours	6.11 g.	
NaOH added for pH control (16.25 ml.)(0.04 g./ml.)	0.65	
Total NaOH	6.76 g.	
Total Na^+ (6.76)(23/40)		3.89 g.

Ion	g.	g./meq.	meq.		
Cl^-	3.52	0.0355	96.2	151.2	approximate check
ClO_3^-	3.69	0.0835	44.2		
SO_4^{2-}	0.52	0.0480	10.8		
Na^+	3.89	0.0230	169.0	169.0	

Salt	meq.	g./meq.	g.
NaCl	96.2	0.0585	5.62
$NaClO_3$	44.2	0.106	4.68
Na_2SO_4	10.8	0.071	0.77

Page 35, Arabinose

Duplicate aliquots containing 3.02 mg. of the carbohydrates extracted from the oxidation solution were diluted with 49.88 and 51.15 mg. of pure arabinose. The specific activity of the original carbohydrates was 15,800 dis./(min.)(mg. glucosyl C) and of the isolated diluted arabinose was 12.0 ± 2.0 and 7.3 ± 2.0 dis./(min.)(mg. C). The arabinose content of the carbohydrate material was therefore 1.26 and 0.78%.

Page 37, M*BG

TABLE XXXIX

SPECIFIC ACTIVITY OF M*BG

Diluted M*BG Sample	Total Carbon, mg.	Net Counts Per Min.	$\frac{V}{r}\frac{E}{}$	Specific Activity[a]
1	0.95	8096 ± 27	0.845	10,085
2	0.95	8088 ± 20	0.8263	10,303
3	0.95	8207 ± 26	0.845	10,223
4	0.95	8108 ± 29	0.8263	10,328
			Average	10,235

[a] dis./(min.)(mg. total C) in the diluted sample.

The M*BG was diluted 1:10 with unlabeled MBG. Therefore, the specific activity of the M*BG was 112,600 dis./(min.)(mg. total C) or 788,200 dis./(min.) per mg. aglucone carbon.

Page 40, Carbon Dioxide

TABLE XL

SPECIFIC ACTIVITY DATA FOR CARBON DIOXIDE

Oxidation Time, hr.	Aliquot of Oxid. Soln. Used, %	Aglucone Carbon in Oxid. Soln., mg.[e]	Net Counts/Min. Found in 2 ml. of NaOH Soln.[a]
1.5	15	30.21	3.4 ± 2.4[b]
4	15	30.05	25.6 ± 3.8[b,c]
9	15	21.64	19.85 ± 3.10[d]
18	5	83.45	97.0 ± 4.1[b]

[a] $\frac{V}{r}\frac{E}{}$ = 0.8263.

[b] Second count, after overnight flow of N_2.

[c] Started with 46 ml. instead of 50 ml. of NaOH in the absorption bottle.

[d] Average of two counts: 2.5 hours (slightly higher count) and 8 hours.

[e] Calculated from Table VI. The specific activity of the carbohydrates is 788,200 dis./(min.)(mg. aglucone C).

Page 40, Methanol

A 20-ml. aliquot of the oxidation solution was taken in each case. Table VI shows the carbohydrate concentrations in the aliquots. The specific activity of this carbohydrate material was 788,200 dis./(min.)(mg. aglucone C). The inactive methanol added for isotope dilution contained 11.88 mg. of carbon in each case. The specific activity of the methyl p-nitrobenzoate obtained by the method in Appendix VI is shown in Table XLI.

TABLE XLI

ISOTOPE DILUTION DATA FOR METHANOL

Oxidation Time, hr.	Ester Activity, dis./(min.)(mg. total C)	
1.5	320.5 ± 6.5	336.0 ± 5.9
4	430.2 ± 6.8	450.7 ± 6.8
9	925 ± 5	884 ± 8
18	1601 ± 12	1629 ± 15
Blank	11.6 ± 1.0	

Page 42, Formaldehyde and Formic Acid

The dilution is explained in Step B, page 118. Table VI shows the carbohydrate concentration of the oxidation solutions. The specific activity of this carbohydrate material was 788,200 dis./(min.)(mg. aglucone C). The carbon dioxide finally produced from both the formaldehyde and the formic acid had the specific activity shown in Table XLII.

TABLE XLII

ISOTOPE DILUTION DATA FROM FORMALDEHYDE AND FORMIC ACID

Oxid. Time, hr.	Carbon Dioxide Specific Activity, dis./(min.)(mg. C)			
	From Formaldehyde		From Formic Acid	
1.5	124.3 ± 3.0	122.4 ± 3.1	28.2 ± 2.3	35.9 ± 1.9
4	212 ± 4.3	201 ± 3.9	52.7 ± 2.1	42.1 ± 1.8
9	469 ± 4.7	460 ± 2.7	80.0 ± 2.5	84.5 ± 2.8
18	954 ± 8.7	918 ± 7.8	157 ± 1.9	120 ± 3.4
Blank	6.7 ± 2.1	4.6 ± 2.6	10.4 ± 1.3	9.9 ± 1.1

Page 43, Hydrolyzable Methanol

The dilution is given on page 121. Table VI shows the carbohydrate concentration in the oxidation solutions. The specific activity of this carbohydrate material is 788,200 dis./(min.)(mg. aglucone C). The per cent of aglucone carbon found as hydrolyzable methanol (Table XI) can be calculated from the specific activities of the methyl p-nitrobenzoate samples obtained by the method of Appendix VI and shown in Table XLIII.

TABLE XLIII

ISOTOPE DILUTION DATA FOR HYDROLYZABLE METHANOL

Oxidation Time, hr.	Specific Activity Methyl p-Nitrobenzoate, dis./(min.)(mg. total C)	
1.5	1486	1564
4	1537	1541
9	1636	1674
18	1673	1668

Page 99, Carbon Dioxide

TABLE XLIV

SPECIFIC ACTIVITY DATA FOR CARBON DIOXIDE

Oxidation Time, hr.	Aliquot of Oxid. Soln. Used, %	Net Counts/Min. Found in 2 ml. of NaOH Soln.	Hours of Nitrogen Flow
9	22.67[a]	9.5 ± 6.0[b,c]	10
		8.0 ± 6.0	12
		7.0 ± 7.0	23
18	10.00	42.0 ± 7.0[c]	9
		44.0 ± 6.0	19
		46.0 ± 7.0	23.5

[a]The carbohydrate concentration in the oxidation solutions is shown in Table XVIII. These carbohydrates had a specific activity of 788,200 dis./(min.)(mg. aglucone C).

[b]Started with 44 ml. instead of 50 ml. in the absorption bottle.

[c]$\frac{V}{r}E = 0.845$.

An average was used for the calculation of the nine-hour sample, and for the eighteen-hour sample the results were extrapolated back to zero hour nitrogen flow. The calculations were made by the method of Table XIX.

Page 100, Methanol

Calculations for the 18-hour sample are shown on pages 107 and 108. The dilution and carbohydrate concentrations in the solutions are shown in Table XVIII. The activity of these carbohydrates was 788,200 dis./(min.)(per mg. of aglucone carbon). The specific activities of the resulting ester is given on page 107. Calculations for the nine-hour solution and the simplified calculation (Footnote 2, page 108) for the eighteen-hour solution may be made with this data and Equation (3), page 13.

Page 115, Table XXV See page 146

Page 125, Table XXVIII

A 10-ml. aliquot of the simulated oxidation solution (Table XXVII) in which the M*BG had a specific activity of 788,200 dis./(min.)(mg. aglucone C) was used. Methanol, 158.4 mg., was added for isotope dilution. The specific activity of the methyl p-nitrobenzoate is shown in Table XLV.

TABLE XLV

ISOTOPE DILUTION DATA FOR HYDROLYZABLE METHANOL

Run Number of Table XXVIII	Specific Activity, dis./(min.)(mg. total C)	
2	1752	1725
3	1576	1574
4	1627	--
5	1561	--
6	1769	1761
7	1648	1845

Page 115, Table XXV

TABLE XLVI

ISOTOPE DILUTION DATA FOR UNREACTED GLUCOSIDE

Sample of Table XXV	Aliquot of Oxid. Soln. Taken, ml.	Carbohydrates in Aliquot, mg.	Dilution with MBG(anhyd.), mg.	Specific Activity[a]
439[b]	10[c]	3.02	45.57	739
440[b]	10[c]	3.02	48.30	728
441[b]	10[c]	3.02	50.05	681
442[b]	10[c]	3.02	48.25	711
455[b]	10	16.26	251.3	739
455(P)[b]				713
456[b]	10	16.26	251.3	741
456(P)[b]				697
495	5	8.475	241.5	3265
495(P)				3239
496	5	8.475	240.7	3281
496(P)				3233
497	5	8.520	242.2	3215
497(P)				3170
498	5	8.520	238.6	3212
498(P)				3229
499[b]	10	18.03	236.6	843
499(P)[b]				845
500[b]	10	18.03	236.8	858
500(P)[b]				849
631	5	8.295	244.5	3226
631(P)				3249
632	5	8.295	257.7	3029
632(P)				3185
633	5	8.165	256.2	2887
633(P)				3167
634	5	8.165	239.4	3345
634(P)				3062
635	5	9.165	239.5	3353
635(P)				3433
636	5	9.165	242.4	3208
636(P)				3629
637	5	9.240	239.0	2923
637(P)				3158
638	5	9.240	236.7	3300
638(P)				3330
654	1	1.659	479.7	371
655	1	1.659	482.2	352
656	1	1.633	478.9	356
657	1	1.633	478.8	355
658	1	1.833	480.0	364
659	1	1.833	479.3	374
660	1	1.848	478.1	350
661	1	1.848	478.7	334

[a] dis./(min.)(mg. total C).
[b] Original MBG* 15,800 dis./(min.)(mg. glucosyl C). In the other runs the original M*BG had 788,200 dis./(min.)(mg. aglucone C).
[c] Extracted oxidation solution.